Titre: Title:	Impact des organismes supérieurs sur la qualité microbiologique de l'eau potable
Auteur: Author:	Françoise Bichai
Date:	2010
Type:	Mémoire ou thèse / Dissertation or Thesis
Référence: Citation:	Bichai, F. (2010). Impact des organismes supérieurs sur la qualité microbiologique de l'eau potable [Thèse de doctorat, École Polytechnique de Montréal]. PolyPublie. https://publications.polymtl.ca/343/

UNIVERSITÉ DE MONTRÉAL

IMPACT DES ORGANISMES SUPÉRIEURS SUR LA QUALITÉ MICROBIOLOGIQUE DE L'EAU POTABLE

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ÉCOLE POLYTECHNIQUE DE MONTRÉAL

THÈSE PRÉSENTÉE EN VUE DE L'OBTENTION

DU DIPLÔME DE PHILOSOPHIAE DOCTOR (Ph.D.)

(GÉNIE CIVIL)

MAI 2010

UNIVERSITÉ DE MONTRÉAL

ÉCOLE POLYTECHNIQUE DE MONTRÉAL

Cette thèse intitulée:

IMPACT DES ORGANISMES SUPÉRIEURS SUR LA QUALITÉ MICROBIOLOGIQUE DE L'EAU POTABLE

Présentée par : BICHAI Françoise

en vue de l'obtention du diplôme de : Philosophiae Doctor

a été dûment acceptée par le jury d'examen constitué de :

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DÉDICACE

À Cindy et Isabelle pour l'incroyable solidarité ☺

REMERCIEMENTS

Une thèse de doctorat peut apparaître comme un document très aride, très froid, rigide, très scientifique évidemment dans notre cas. Le travail d'une thèse représente effectivement des heures de travail difficile, solitaire; le milieu du laboratoire est empreint de restrictions, de règles, de rigueur dans les manipulations, même dans la tenue vestimentaire. Pourtant, le doctorat en soi est loin d'être dénué de vie, de créativité et d'interactions humaines. Il s'agit avant tout d'un parcours très humain, un projet plein d'influences et d'entraves, de réflexions, d'évolution et de liens tissés, transformés. En effet, l'entreprise d'un doctorat en est une de longue haleine et est aussi une épopée souvent parcourue à l'une des époques les plus belles, riches et vivantes de notre évolution comme étudiant, comme jeune adulte et futur professionnel. L'apprentissage des études doctorales trouve une part précieuse de sa valeur en le fait qu'il est enrichi des parcours parallèles de nos collègues, de nos superviseurs, de nos collaborateurs et aussi de nos proches…

Cette section de remerciements est à mon avis la plus satisfaisante à écrire de toute ma thèse. J'ai beaucoup de reconnaissance et d'admiration à témoigner d'abord à mon cher directeur de recherche de toujours, le Dr. Benoit Barbeau, qui est un modèle professionnel pour sa gestion calme de son temps pourtant si chargé de responsabilités importantes et bien sûr pour sa maîtrise remarquable de notre domaine, à la fois comme ingénieur et comme professeur, pédagogue et comme chercheur. (J'en profite Benoit puisque ce n'est pas un article scientifique (enfin!) pour faire des longues phrases…) Benoit, merci pour ta disponibilité, ta sensibilité aux contextes personnels à travers lesquels tes étudiants tentent d'atteindre tous les objectifs d'un doctorat, merci pour ton ouverture à nos caractères et à notre touche propres en recherche, merci pour la liberté que tu nous laisses en nous témoignant ta confiance dans notre travail. Ce sont cette confiance et cette liberté qui, à mon avis, nous laissent spontanément faire ressortir le meilleur de nous-même dans notre travail et comme personnes. Merci de reconnaître nos efforts et nos difficultés. Merci pour la relation que nous avons pu développer depuis mon entrée à la chaire comme stagiaire, merci aussi de m'avoir dirigée et encouragée à passer au doctorat (malgré mes premières réticences!) et d'avoir ainsi joué un rôle majeur dans ma découverte de possibilités de carrière qui me motivent vraiment; merci de m'avoir soutenue dans ma collaboration avec les

chercheurs de KWR aux Pays-Bas, merci pour ton efficacité qui facilite toujours le lourd processus de la publication d'articles! Merci d'avoir aussi laissé souvent libre cours à ma créativité dans mes présentations et conférences, merci d'avoir même accepté à mon grand étonnement de présenter si honorablement mes BD (thérapeutiques?) de nématodes au grand public du WQTC à Cincinatti en mon absence! Merci aussi d'avoir progressivement laissé s'infiltrer un sujet comme la désinfection solaire dans ma recherche et même dans tes questions d'examen! Merci enfin pour ton soutien incroyablement solide, attentif, fiable, cordial dans mon *sprint* final des dernières semaines… Tu as beaucoup facilité la longue traversée de mes années de doctorat, comme d'ailleurs mes chers collègues de bureau avec qui j'ai tissé des liens plus que professionnels.

Isabelle! Cindy!!!! Arash! J'ai été très heureuse de partager votre bureau! (Arash, tu nous manques avec tous tes séjours en Australie!) Merci pour tous les thés, lunchs, gâteaux, amandes au chocolat partagées, merci pour votre ouverture à n'importe quelle musique sur mon ordinateur! C'est vous qui avez définitivement lancé ma carrière de DJ Frankie! Merci pour le bel esprit de solidarité dans le bureau, de soutien et de compréhension, de sensibilité envers les autres, d'encouragements mutuels, merci pour tout le côté social et l'humour qui rend la vie tellement plus légère au quotidien! J'ai apprécié toutes nos discussions (majoritairement non professionnelles!) et la créativité sur notre tableau noir. Ce fut une chance de partager avec vous des capsules de *PHDcomics* et bien sûr des cocktails exotiques sur les terrasses quand il faisait beau (et on a encore tout l'été!)…! Les filles, Majdala aussi (notre chère princesse des eaux usées), vous êtes devenues des amies importantes que je suis vraiment triste de quitter comme collègues. Vous tous qui avez tellement embelli mes dernières années à la Polytechnique, chères Murielle et Ana aussi, j'espère toujours qu'on se suivra de près ou de loin dans la suite de notre carrière et qu'on se refera souvent des weekends de ski de fond ou de nos fameux soupers, des brunchs de filles interminables… (D'ailleurs je vous attends toutes en Espagne en 2011…! vue sur la mer.. ☺)

Merci à la belle Michèle Prévost, notre grande chef à tous, notre inspiration, notre moteur et notre tornade, avec un vrai cœur de mère! Ce fut un privilège de te connaître un peu mieux à Amsterdam, toi et ton cher mari, vous êtes un si beau couple, chaleureux et rafraîchissant à la

fois! Michèle tu es un bel exemple de réussite pour nous, tes étudiantes, qui tentons d'absorber un peu de ton énergie fonceuse qui déstabilise toute l'industrie de l'eau potable !

Merci aux autres professeurs de la chaire, à Raymond Desjardins qui m'a fait faire mes tous premiers pas dans le domaine du traitement des eaux… Merci à mon co-directeur Pierre Payment, pour votre contribution précieuse en tant que microbiologiste à mon arrivée dans ce projet, avec ma formation d'ingénieure civil et une touche d'insécurité, légèrement intimidée d'ailleurs (!), merci pour votre regard plein d'assurance et de sagesse sur mon travail et mon parcours, pour votre délicatesse et votre courtoisie dans nos rapports, merci d'avoir partagé un peu de votre grande expérience en recherche avec moi, merci pour les souvenirs -vidéo de Naxos! Merci à Annie Locas, qui a amené la première les nématodes du réseau de Montréal sous les projecteurs, un point de départ important pour ma recherche…! Merci aussi au Dr. Pierre Servais, c'est un honneur de vous avoir comme membre externe sur ce jury, merci pour vos visites de Belgique, pour votre intérêt envers ma recherche et pour les relations amicales que vous entretenez avec notre chaire de recherche, ce fut un plaisir à chaque fois de partager des repas avec vous dans différentes villes du monde (!), en espérant poursuivre les bonnes habitudes... (Merci tout spécialement pour les inoubliables moules-frites à Bruxelles!) Merci à notre post-doc de passage Normand Labbé, qui a assisté à mes débuts comme nouvelle étudiante au doctorat dans le laboratoire du 6^{e} (un véritable terrain de jeu pour lui, un monde parallèle plein de dangers et de perplexité pour moi), merci pour ton *training* très pédagogique entre autre sur la méthode FISH et pour ton assistance dans le démarrage de mon aventure avec les nématodes... Merci au Dr. Serge Parent d'avoir accepté de siéger sur ce comité comme évaluateur externe pour ma thèse, merci pour vos conseils d'expert des nématodes, pour votre enthousiasme et votre aide toujours très généreuse dans mes débuts surtout, sur un sujet qui me semblait si étranger…

Beste Wim.. (Dr. Hijnen!) Dank je wel en vriendelijke groeten ! Ik zal.. at least remember those words from my Dutch experience. It was a true pleasure and an incredible privilege to work with you and be your co-author on a few papers and conferences as an important part of my thesis. It was great for me to have the opportunity to collaborate with you on some of your on-going experiments in the Netherlands. I respect and admire your work both for its remarkable scientific value and for the way you are conducting it and reflecting upon it, with a thoughtful perspective

based on such an obviously solid experience, an impressive amount of knowledge and professional wisdom (?!). Thank you so much for keenly undertaking this collaboration with me, thank you for our first fortuitous talk at the WQTC in Denver in 2006! I was so happy (and impressed!) to meet you! Going to your country was an amazing and important experience, both on professional and personal sides. Thank you for your careful supervision of my work for 8 months, for all your ideas, suggestions, your help in many ways, for your constant courtesy and concern for my well-being in my Dutch life. Thank you very much for your warm welcome in your family as well. Thank you for making my stay at KWR very enriching and comfortable. I really hope that we will remain in contact on the basis of this honest friendship which I feel very lucky to have gained and (who knows?) maybe for research collaboration again.

Thank you as well to my dearest Gemma whose office I delightfully shared at KWR. She is and will remain officially my young and sparkling Dutch mother and a true friend. Thank you to all my colleagues of the microbiology group at KWR and the staff from the lab (special thanks to Anke who taught me so much!). Many thanks to Dr. Gertjan Medema, thank you for accepting me inside your Microbiology group for 8 months, it was fascinating to witness the great work and high qualification of your team; I am amazed with your success as a scientist *and* a musician! Thank you for your songs (I'll sure remember the Dutch *Gents* advice to *believe in yourself*!).. Thanks also to Patrick for all the music which keeps my memories of Holland alive on a day to day basis! Thank you also to Yolanda Dullemont and Eric Baars from Waternet and Martine Rosielle from HWL for their kind and helpful contribution in my work in the Netherlands. Thank you very much Eric for the nice tours around Amsterdam's treatment plants!

Merci à tout le personnel des laboratoires de la Chaire, Jacinthe, Julie, Mélanie que j'ai connues d'abord comme stagiaire d'été en 2003, qui m'ont vue de près débuter timidement dans un laboratoire, merci pour votre gentillesse constante depuis toujours, Mireille, Yves, Marcellin, Annie et Shokoufeh, Marie-Claude… France, notre soleil du 4^{e}… Merci pour ton rire, ta spontanéité et tes attentions… Merci à tous les autres étudiants de la chaire, merci à Simon L. « *mon* stagiaire », Anne-Sophie (et tous nos concerts!), Clément, Élise, Gabrielle (Little Tiger!), notre nouvelle venue Céline, aux autres qui ont gradué de la maîtrise au fil des années et avec qui

on a eu de très bonnes soirées à saveur encore étudiante, Natasha, Pierre, Guillaume, Daniel, Simon V., Romain Broséus (the one and only!), je suis très heureuse de garder le contact…!

Merci aussi à Manon Latour et Lilia Paquin qu'il est un plaisir de côtoyer, merci de nous répondre toujours de manière si serviable et sympathique, merci pour l'efficacité et la bienveillance avec laquelle vous supervisez tous les détails administratifs de nos activités aux études supérieures.

Merci à Sébastien aussi… avec qui j'ai fini mon bac à poly et commencé ma maîtrise, qui m'a encouragée dans mon passage au doctorat, qui m'a soutenue à travers l'épreuve ultime (!) de l'examen de synthèse et qui m'a laissée partir aux Pays-Bas, saisir une occasion qui me tenait à cœur malgré ce que ça lui en coûtait. Merci d'avoir voulu souvent penser à moi avant tout, à mon bonheur, merci pour toutes les années de soutien et de partage, j'en garde un souvenir de belle complicité et de sincère dévouement. Tu as longtemps été mon réconfort le plus présent, le plus proche, le plus constant.

Merci à ma famille élargie (québécoise, égyptienne, française, à mes chères cousines, chers cousins, oncles et tantes à Montréal et ailleurs dans le monde), mais surtout merci à mon père (le Sphinx! toujours calme, fort, solide à travers les tempêtes de sable… le roc qui soutient notre famille, papa je suis très fière d'être ta fille, c'est une chance immense de t'avoir dans ma vie, tu es un cadeau et un épatement pour tous ceux qui ont le privilège de te côtoyer) et mes deux sœurs Nathalie et Judith que j'adore (et qui aiment beaucoup exagérer mon succès professionnel!), qui ne me laissent jamais douter de leur amour («*as a sister, which is a relationship stronger than marriage*» pour citer notre film culte!), merci pour votre confiance en moi presque inébranlable (!) à travers mes parcours un peu épiques, merci à leurs deux merveilleux copains Pascal et Bruno qui complètent notre famille en y apportant leur énergie et leur humour, merci pour vos encouragements sincères. Merci à Jeannine, si radieuse, généreuse, positive, confiante, merci d'avoir épousé mon père et de l'avoir rendu plus fort, plus heureux, plus vivant. C'est grâce à vous tous que les fameux soupers du dimanche soir se transforment à chaque fois en une soirée haute en couleurs et en divertissement, merci papa et Jeannine pour votre générosité et votre perpétuel accueil dans une ambiance toujours si confortable et si détendue…!

Merci à ma mère, Denise Poirier, décédée le 22 juin 1998, qui m'a élevée avec amour jusqu'à mes 16 ans, jusqu'au bout de ses forces, avec sa force de caractère, son courage, sa sensibilité, sa musique, et qui m'a fait grandir malgré elle par l'immense épreuve qui l'a emportée et qui nous a à tout jamais secoués, marqués, changés. Je sais que tu aimerais lire ma thèse, je sais que tu aurais aussi aimé faire de la recherche, je sais que tu serais contente pour moi, fière, intéressée, j'aurais tellement aimé fêter cet accomplissement avec toi et vivre avec toi toute ces prochaines étapes que je ne devine pas encore... Chère maman, je serai heureuse pour compenser toute ta souffrance…

Merci à tous mes amis qui ne liront jamais cette thèse (je vous comprends!) : Hélène (ma deuxième famille, tu es géniale, merci d'être toujours là, presque inconditionnellement, à travers tes déplacements aux quatre coins du Québec et du monde (!), ta générosité m'épate encore toujours après 23 ans d'amitié ! je t'adore), ma chère Daria (merci pour ton excentricité, ton art, ton ouverture, ta sagesse –oui oui!- ta chaleur et ton amitié depuis littéralement l'autre bout du monde, vivement que tu reviennes à Montréal!), Myriam (merci pour tous les weekends, Toronto, les concerts, les longs cafés, ton support moral et tes opinions franches à travers mes téléromans, ta cyber-présence (!) et surtout merci pour tous les rires!), chère Émilie de l'autre côté de la rivière (merci pour tous les soupers, les bonnes conversations, les sorties et toute la vaisselle! ☺), Ben (la perle de mon bac! merci d'être un presque-frère Bichai, merci pour tes visites rituelles du 24 décembre!), ma chère cousine Lorraine (pour tous nos souvenirs ensemble, pour notre profonde complicité, merci d'être une cousine si proche peu importe où on est dans le monde!), Avinash (Nashou!!! merci pour le *free-style*! pour tous les weekends en Europe en 2009, les inspirations artistiques et encore toutes les vacances à venir, merci pour ton suivi continu depuis Paris incluant un envoi postal magique (!) à travers cette finale très intense de ma thèse!). Vous avez toujours été, au-delà de toutes mes attentes, un soutien, un apaisement, un défoulement, une énergie, des voyages, beaucoup de rires et de réconfort; merci pour votre loyauté, votre générosité et tout le bien que vous me reflétez souvent. Merci JF d'être venu par hasard à Utrecht! Merci pour toutes les soirées, merci d'avoir tout rendu tellement plus facile là-bas, plus drôle, plus social, plus dynamique, plus mémorable..! Gracias Sebastián (por todo, por los 'paella dinners', por Ecu y sobre todo por sacarme de la funda!), merci Yapo (pour le torrent de musique (!) qui a donné un thème à ma dernière année de doctorat, aux changements de saisons,

pour *Flowers in December*, *Ojos de Brujo*, je te souhaite un doc (?) et une carrière de rêves..!).. Merci aux membres de mon groupe de musique (Tom, Édith, Pasc, Colin) depuis les années de bac à poly et à travers mes quelques allers-retours à l'étranger (désolée), merci de garder l'Ombudsband en vie (!) pour longtemps encore j'espère… Merci à ma professeure de violon Jean McRae, à mes professeurs d'arts et à tous ceux qui sont venus voir des concerts avec moi, qu'est-ce que je ferais sans vous dans un monde trop scientifique..?!

Je remercie aussi évidemment le CRSNG et les partenaires industriels de notre chaire de recherche, les villes de Montréal et Laval et John Meunier inc. pour leur soutien financier indispensable à la réalisation de nos travaux. Merci au CRSNG pour ma bourse d'études doctorales…

PS. Je voulais juste être sûre d'avoir bien remercié mon directeur Benoit… après ce flot de remerciements éparpillés, merci encore Benoit et bonne suite!

RÉSUMÉ

Les organismes supérieurs commencent à être perçus comme une préoccupation sanitaire dans l'eau potable puisqu'ils sont de plus en plus décrits comme de potentiels véhicules de microorganismes pathogènes internalisés et, jusqu'à un certain point, comme des boucliers de protection contre la désinfection pour les microorganismes qu'ils transportent.

Les travaux de cette thèse se concentrent sur l'étude des organismes supérieurs comme hôtes et véhicules de microorganismes pathogènes dans l'eau potable, en considérant principalement le zooplancton multicellulaire tel que les rotifères et le zooplancton crustacéen, ainsi que les nématodes, et excluent essentiellement l'étude des amibes, qui sont déjà qualifiées de « cheval de Troie » des microorganismes et sont davantage documentées à cet égard.

L'objectif général de cette thèse de doctorat consiste à évaluer l'impact de l'internalisation des microorganismes pathogènes par les organismes supérieurs sur le risque microbiologique lié à la consommation d'eau potable.

La réalisation de cet objectif général nécessite de considérer plusieurs aspects du phénomène qui sont encore peu documentés dans la littérature scientifique, soit principalement: (i) la probabilité d'occurrence d'un microorganisme pathogène à l'intérieur d'un organisme supérieur dans l'eau potable; (ii) l'impact des procédés de désinfection sur la survie d'un microorganisme internalisé par un organisme supérieur.

Plus précisément, cette thèse se donne pour objectifs spécifiques de :

(1) caractériser l'occurrence du phénomène d'internalisation de microorganismes pathogènes par les organismes supérieurs dans la filtration granulaire et le transport des organismes internalisés dans l'eau filtrée;

(2) caractériser l'effet de protection dont bénéficient les microorganismes internalisés par des organismes supérieurs sur la performance de la désinfection UV;

(3) comparer l'effet de protection dû à l'internalisation des microorganismes parallèlement à celui de l'agrégation et de l'attachement aux particules sur la performance de deux

procédés voisins, soit la désinfection UVC et la désinfection solaire (UVA), appliqués respectivement dans les pays industrialisés et en développement;

(4) développer, par la caractérisation d'une chaîne d'événements décrivant l'internalisation et le transport de microorganismes par les organismes supérieurs, un modèle d'analyse de risque permettant de quantifier le risque sanitaire associé aux microorganismes pathogènes internalisés dans l'eau potable.

Dans cette thèse, la filtration granulaire a été ciblée comme un milieu favorable à l'internalisation des microorganismes, entre autres parce que les organismes supérieurs y sont abondants et que leurs activités de prédation y sont intensifiées. L'internalisation et le transport des (oo)cystes de *Cryptosporidium* et *Giardia* dans la filtration granulaire ont été étudiés en première phase de cette thèse. Une étude a été réalisée à l'échelle pilote en ensemençant de hautes concentrations d'(oo)cystes de *Cryptosporidium* et *Giardia* préalablement inactivés aux UV dans des colonnes de filtration au charbon actif granulaire (CAG) naturellement colonisées de zooplancton. Un protocole expérimental a été développé afin de permettre la libération et l'énumération des (oo)cystes internalisés suite à une rupture complète d'organismes du zooplancton par sonication. Des échantillons de matériau granulaire (CAG) ont été prélevés à différentes profondeurs du lit filtrant suite à l'ensemencement des filtres. Le zooplancton a été isolé des échantillons de CAG ainsi qu'à l'effluent filtré dans le but d'extraire et d'énumérer les (oo)cystes internalisés. Dans ces conditions, il a été démontré que la prédation par le zooplancton résulte en l'internalisation d'une fraction limitée des (oo)cystes dans le lit filtrant, suivie du transport d'une portion des (oo)cystes internalisés à l'effluent. Une hausse de la concentration d'(oo)cystes internalisés à l'effluent filtré a été observée 3 semaines après l'ensemencement des filtres en l'absence de rétrolavage. Les rotifères sont suggérés comme principaux responsables du transport des (oo)cystes internalisés à l'effluent.

En deuxième phase de ce projet, l'impact de l'internalisation des microorganismes sur l'efficacité de la désinfection UV a été évalué en utilisant le nématode *Caenorhabditis elegans* comme prédateur pour les bactéries *Escherichia coli* et les spores de *Bacillus subtilis*. Un protocole a été développé afin de (i) permettre la prédation et l'internalisation des deux microorganismes ciblés

par les nématodes, (ii) exposer les co-suspensions à l'irradiation UV (254 nm) et (iii) extraire les bactéries internalisées grâce à un protocole de sonication permettant la rupture des nématodes et l'énumération des bactéries par des méthodes de culture standards. Ces travaux ont permis de démontrer que les microorganismes internalisés par des organismes supérieurs peuvent être partiellement protégés contre la désinfection UV (254 nm). Environ 15-16% de la fluence de 40 mJ/cm^2, typiquement appliquée dans les usines de traitement d'eau potable, atteint les bactéries *E. coli* et les spores de *B. subtilis* internalisés par des nématodes *C. elegans*. Cet effet protecteur est moins accentué face à une fluence plus faible. Ainsi, la désinfection UV présente un potentiel d'inactivation des microorganismes internalisés par des organismes supérieurs.

Suite à ces travaux, une évaluation comparative des mécanismes de protection microbiens dans la désinfection UV (UVC 254 nm) et solaire (UVA) a été réalisée. Deux séries d'essais ont été entreprises afin d'évaluer l'impact de deux types de mécanismes de protection face à la désinfection solaire, soit (i) l'agrégation des microorganismes et l'attachement aux particules et (ii) l'internalisation des microorganismes par les organismes supérieurs. Dans le premier cas, les travaux ont été réalisés sur la base d'un protocole de désinfection préalablement développé pour évaluer l'impact de la turbidité dans la désinfection UVC (Caron et al. 2007): des échantillons d'eau de rivière ont été traités de manière plus ou moins extensive afin de distinguer l'impact de la dispersion des agrégats et de l'enlèvement de particules (par filtration) sur l'efficacité de la désinfection UVA des coliformes totaux indigènes. Les essais sur l'internalisation ont été exécutés à l'aide du protocole développé pour les essais UVC dans le cadre de cette thèse, avec *E. coli* comme cible de la désinfection internalisé par le nématode *C. elegans*. L'irradiation UVA a été simulée à l'aide d'une lampe (365 nm) en laboratoire. Une réduction du taux d'inactivation des coliformes totaux par les UVA due à l'agrégation des microorganismes entre eux a été démontrée, soit un effet protecteur semblable à celui rapporté face à la désinfection UVC. Toutefois, l'enlèvement des particules par une filtration membranaire (8 μm) n'a démontré aucun impact sur l'efficacité de l'inactivation par les UVA, contrairement à la hausse du taux d'inactivation démontré par un protocole identique face à l'inactivation UV à 254 nm. L'internalisation par les nématodes a démontré un effet protecteur comparable face à la désinfection UVA et UVC. En effet, environ 24% d'une fluence UVA de 5.60 J/cm^2 a été estimée effective pour inactiver les bactéries *E. coli* localisées à l'intérieur de nématodes *C.*

elegans. Comme en désinfection UV (254 nm), une protection moins prononcée a été observée à une fluence plus faible.

Enfin, en dernière étape de cette thèse, une analyse quantitative du risque microbien a été réalisée afin d'évaluer la probabilité annuelle d'infection chez les consommateurs due aux microorganismes pathogènes internalisés par des organismes du zooplancton dans l'eau potable. Dans cette étude, la prédation des (oo)cystes de protozoaires par les rotifères dans la filtration granulaire a été ciblée comme origine du risque d'internalisation des microorganismes dans l'eau potable. Des simulations Monte-Carlo ont été exécutées à l'aide du logiciel Crystal Ball® (Decisioneering, USA). La quantification de la plupart des variables impliquées dans le calcul du risque par le modèle développé a été dérivée de données générées à l'échelle pilote dans l'étude de filtration décrite dans cette thèse. Cette analyse de risque a permis d'estimer que le risque annuel d'infection chez les consommateurs d'eau potable dû à l'ingestion d'(oo)cystes de protozoaires internalisés par des organismes supérieurs est inférieur au risque permis d'une infection par 10 000 personnes annuellement. L'application d'un procédé de désinfection UV en aval de la filtration granulaire dans les usines de traitement d'eau potable a été évaluée comme pouvant réduire d'environ 2 ordres de magnitude la probabilité d'infection associée aux (oo)cystes internalisés dans l'eau potable.

Bien que, suite aux travaux de cette thèse, le risque sanitaire associé aux microorganismes internalisés dans l'eau potable soit supposé faible, plusieurs aspects de la question gagneraient encore à être davantage élucidés. D'abord, les mesures d'organismes internalisés présentées dans l'étude de filtration à échelle pilote fournissent des données quantitatives nouvelles, mais qui demandent à être renforcées par des mesures expérimentales similaires. Il est suggéré d'envisager de telles études au niveau de la filtration lente, qui présente un haut potentiel d'internalisation dû à la densité du zooplancton dans la partie supérieure du lit filtrant et l'importance de la prédation dans ce type de filtration granulaire. Des travaux futurs devraient également considérer une étude plus approfondie du risque associé au transport de bactéries ayant un potentiel d'amplification dans le tube digestif de l'hôte. En effet, une telle capacité d'amplification des microorganismes internalisés suggère un risque sanitaire potentiellement plus élevé que celui évalué dans cette thèse pour des (oo)cystes de protozoaires internalisés. La

conduite d'échantillonnages environnementaux visant la détection de microorganismes naturellement internalisés par le zooplancton est également recommandée. Enfin, au niveau de l'industrie de l'eau potable, il est suggéré, suite aux travaux de cette thèse, de considérer le rôle potentiel des organismes supérieurs dans le transport et la protection de microorganismes internalisés comme pouvant être à l'origine d'une détection occasionnelle ou d'une persistance inattendue d'un microorganisme pathogène ou indicateur à certaines étapes du traitement en usine ou encore en réseau de distribution.

ABSTRACT

Higher organisms are increasingly perceived as a sanitary concern in drinking water as they are described as potential vehicles and, to some extent, as shields against water disinfection for internalized pathogens.

This thesis focuses on the study of higher organisms as hosts and vehicles for pathogenic microorgansims in drinking water, by considering mainly multicellular zooplankton such as rotifers and crustacean zooplankton as well as nematodes, and essentially exclude the study of amoebae, who are already qualified as "Trojan horse" of microorganisms and are more documented in this regard.

The general objective of this thesis is to assess the impact of the internalization of pathogens by higher organisms on the microbiological risk associated with consumption of drinking water. Achieving this objective requires considering several aspects of the phenomenon which are still poorly documented in scientific literature, primarily: (i) the occurrence probability of internalized pathogens within higher organisms in drinking water, and (ii) the impact of disinfection processes on the survival of microorganisms internalized by higher organisms.

More specifically, the specific objectives of this thesis are:

(1) to characterize the occurrence of the phenomenon of pathogens internalization by higher organisms in granular filtration and the transport of internalized organisms in the filtered water;
(2) to characterize the protection effect provided to microorganisms internalized by higher organisms on the performance of UV disinfection;

(3) to compare the protective effect due to internalization of microorganisms and that of aggregation and attachment to particles on the performance of two close processes, UVC disinfection and solar (UVA) disinfection, respectively applied in industrialized and developing countries;
(4) to develop, by characterizing a chain of events describing the internalization and transport of

microorganisms by higher organisms, a QMRA model to quantify the health risk associated with internalized pathogens in drinking water.

In this thesis, granular filtration has been targeted as an environment favourable to the internalization of microorganisms, mainly because higher organisms are abundant and their predation activities are intensified. Internalization and transport of *Cryptosporidium* and *Giardia* (oo)cysts in granular filtration were studied in the first phase of this thesis. A study was conducted at pilot scale by seeding high concentrations of *Cryptosporidium* and *Giardia* (oo)cysts previously inactivated with UV in granular activated carbon (GAC) filtration columns naturally colonized by zooplankton. An experimental protocol has been developed to allow the release and the enumeration of internalized (oo)cysts following a complete disruption of zooplankton organisms by sonication. Samples of granular media (GAC) were collected at different depths of the filter bed after the seeding. Zooplankton was isolated from the GAC samples and from the filtered effluent in order to extract and enumerate internalized (oo)cysts. Under such conditions, it was demonstrated that predation by zooplankton results in the internalization of a limited fraction of (oo)cysts in the filter bed, followed by the transport of a portion of the internalized (oo)cysts to the effluent. An increased concentration of internalized (oo)cysts in the filtered effluent was observed 3 weeks after seeding in the absence of filter backwashing. Rotifers are suggested as the main zooplankton group responsible for the transport of internalized (oo)cysts to the effluent water.

In the second phase of this project, the impact of the internalization of microorganisms on the effectiveness of UV disinfection was evaluated using the nematode *C. elegans* as a predator for *E. coli* bacteria and spores of *B. subtilis*. A protocol was developed to (i) allow predation and internalization of both target microorganisms by nematodes, (ii) expose the co-suspension to UV irradiation (254 nm) and (iii) extract the internalized bacteria using a sonication protocol allowing the disruption of nematodes and enumeration of bacteria by standard culture methods. These assays have demonstrated that microorganisms internalized by higher organisms can be partially protected against UV disinfection (254 nm). Approximately 15-16% of the 40 mJ/cm^2 fluence, typically applied in drinking water treatment plants, reached the *E. coli* bacteria and *B. subtilis* spores internalized by nematode *C. elegans*. This protective effect was less pronounced at a lower

fluence. Thus, UV disinfection presents a potential for inactivation of microorganisms internalized by higher organisms.

Following this work, a comparative evaluation of microbial protective mechanisms in UV (UVC 254 nm) and solar (UVA) disinfection was conducted. Two sets of assays were undertaken to assess the impact of two types of protection mechanisms in solar disinfection, either (i) the aggregation of microorganisms and attachment to particles and (ii) internalization of microorganisms by higher organisms. In the first case, the experimental work was performed on the basis of a disinfection protocol previously developed to assess the impact of turbidity in UVC disinfection (Caron et al. 2007): samples of river water were treated more or less extensively to distinguish the impact of the dispersion of aggregates and particle removal (by filtration) on the effectiveness of the UV inactivation of indigenous total coliforms. Assays on internalization were performed using the protocol developed for UVC tests within this thesis, with *E. coli* as a disinfection target internalized by the nematode *C. elegans*. UVA irradiation was simulated using a lamp (365 nm) in the laboratory. A reduction in the UVA inactivation rate of total coliforms due to aggregation was demonstrated, thus a protective effect similar to that reported against UVC disinfection. However, the removal of particles by membrane filtration (8 μm) showed no impact on the efficiency of inactivation by UVA, in contrast to the raise in inactivation rates found when using an identical protocol to with UVC inactivation. Internalization by nematodes showed a similar protective effect against UVA and UVC disinfection. Approximately 24% of a 5.60 J/cm^2 UVA fluence was estimated to be effective in inactivating *E. coli* bacteria located within the nematode *C. elegans*. Like UVC disinfection (254 nm), a less pronounced protection effect was observed at a lower fluence.

Finally, as a final stage of this thesis, a quantitative microbial risk assessment was performed to quantify the annual probability of infection among drinking water consumers due to pathogens internalized by zooplankton organisms. Predation on protozoan (oo)cysts by rotifers in granular filtration has been targeted as the source of the risk of microorganisms' internalization in drinking water. Monte-Carlo simulations were performed using the software Crystal Ball® (Decisioneering, USA). Quantification of most of the variables involved in calculating the risk in the developed model was derived from data generated in the pilot-scale filtration study described in this thesis. This risk analysis allowed estimating that the annual risk of infection among drinking water consumers due to internalized (oo)cysts is lower than the targeted risk of one

infection in 10,000 people annually. The application of a UV disinfection process following granular filtration in drinking water treatment plants was estimated to potentially reduce by about two orders of magnitude the probability of infection associated with the internalized (oo)cysts in the treated water.

Although following the work of this thesis the health risk associated with internalized microorganisms in drinking water is assumed to be low, several aspects of this issue would benefit from further elucidation. First, internalized organisms measurements presented in the pilot-scale filtration study provide new quantitative data, which need however to be reinforced by similar experimental measurements. It is suggested to consider undertaking such studies with slow sand filtration, which has a high potential for internalization due to the density of zooplankton near the surface of the filter bed and the importance of predation in this type of granular filtration. Future work should also consider a more in-depth assessment of the risk associated with the transport of those bacteria that have a potential for amplification in the gut of their host. Indeed, such a capacity for amplification of internalized organisms suggests a health risk potentially higher than that estimated in this thesis for internalized protozoan (oo)cysts. Conducting environmental sampling for the detection of microorganisms naturally internalized by zooplankton is also recommended. Finally, at the level of the drinking water industry, it is suggested, following the work of this thesis, to consider the potential role of higher organisms in the transport and protection of internalized microorganisms as a possible cause and explanation for occasional detection or unexpected persistence of a pathogenic or indicator microorganism at certain stages of the treatment plant or distribution network.

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CAG	Charbon actif granulaire (GAC)
COT	Carbone organique total (TOC)
CT	Dose de désinfectant chimique correspondant au produit de sa concentration (en mg/L) et du temps de contact (en min)
DEC	Capacité d'élimination décimale (Decimal elimination capacity)
FISH	Hybridation fluorescente *in situ* (Fluorescent *in situ* hybridation)
QMRA	Analyse quantitative du risque microbien (Quantitative microbial risk analysis)
UV	Ultraviolet

AVANT-PROPOS

« L'imagination est plus importante que le savoir. », a dit Albert Einstein.. ☺

On remarque souvent qu'il suffit d'être confronté un jour à notre ignorance sur un sujet pour s'apercevoir rapidement par la suite et à de répétitives occasions, non provoquées, que ce sujet en question fait partie de notre environnement dans une part étonnante bien qu'on ne l'ait jusque là jamais remarqué. L'exemple le plus facile pour moi est la découverte hasardeuse d'un nouveau groupe de musique, par une coïncidence des plus banales qui fait qu'on tombe un jour sur une page de journal ou une affiche au milieu de notre trépidante grande ville. Souvent dans les jours et semaines qui suivent, cette image à peine consciente sera rappelée à notre mémoire évasive en découvrant tour à tour que ce groupe de musique est en pleine effervescence, que ses albums des 5 dernières années sont excellents, qu'ils offrent régulièrement des concerts à Montréal et que l'ordinateur d'un de nos collègues abrite discrètement plusieurs morceaux de leur répertoire.

Dans mon cas, deux découvertes à l'image de ce curieux phénomène aléatoire sont au cœur de mon doctorat : les organismes supérieurs dans l'eau potable et les Hollandais. Il suffit d'accorder un peu d'attention au peuple et à la culture hollandaise pour se rendre compte des innombrables ramifications de leur influence à travers le monde, et oui, même ici au Québec et au Canada, en commençant évidemment par les tulipes d'Ottawa, la *Grolsh* toujours présente dans nos dépanneurs, le film sur Van Gogh au cinéma *Imax*, nos compétiteurs en patinage de vitesse aux Jeux Olympiques, le mari de notre directrice Michèle, les biscuits *Stroopwafels* qui se trouvent à l'épicerie du coin… Les Hollandais ont même déjà occupé l'île Maurice, mon idylle, avant les Français et les Britanniques. En eau potable, ils sont parmi les Européens les plus visibles dans les conférences américaines, les plus avancés dans le domaine de l'analyse de risque (QMRA), les fervents défenseurs d'une eau non chlorée et de qualité exceptionnelle, ils sont très présents sur plusieurs continents comme consultants et entrepreneurs, à tout hasard par exemple en Égypte, pays mythique et spectaculaire de mes ancêtres paternels (les Pharaons ☺)… Depuis mon séjour aux Pays-Bas dans le cadre de ma thèse, la participation des Hollandais dans de multiples sphères d'activités de notre monde ne passe plus inaperçue à mes yeux. C'est lors

d'une présentation devant mes collègues hollandais justement, du groupe de microbiologie de KWR, que j'avais pensé faire le parallèle entre l'implication des organismes supérieurs dans l'eau potable et la révélation de l'influence des Hollandais dans le monde à mes yeux nouvellement intéressés par ce peuple, récemment plus attentifs à leurs apparitions et retombées partout autour de moi. Il s'agit seulement d'éveiller une fois notre attention sur un sujet pour diriger sans le savoir notre curiosité vers d'autres empreintes, d'autres indices et tenter éventuellement de corriger un peu de notre astigmatisme. De la même manière, les organismes supérieurs dans l'eau potable pourraient se révéler influents dans plusieurs situations une fois qu'on leur aurait porté suffisamment d'attention pour les intégrer dans notre banque de connaissances professionnelles qu'on parcourt mentalement, dans un processus souvent désordonné et non systématique, lorsqu'on cherche une solution, une piste ou une nouvelle approche d'un problème dans notre champ d'expertise, l'eau potable. Dans le monde microbien, le discernement des différents auteurs, acteurs de phénomènes observés, s'avère plus délicat ou plus mystérieux, puisqu'on n'y trouve pas de signature officielle, pas de service de traduction, pas de droits d'auteur, pas de politique. Il nous faut donc sans doute, comme scientifique, approcher de plusieurs manières chaque problème pour avoir une chance de retracer les véritables liens de causes à effets à partir des seuls indices observables par les humains, à travers leurs erreurs et dans les limites des méthodes et techniques disponibles. Dans l'étude des phénomènes microbiologiques liés à la qualité de l'eau potable, à travers différents procédés de traitement et de purification, les organismes supérieurs ont longtemps été vastement ignorés, oubliés, ou alors écartés des pistes de solution, négligés et déresponsabilisés comme acteurs pouvant influencer ce résultat vers lequel tous les yeux des experts de la désinfection de l'eau sont tournés, soit la qualité microbiologique de l'eau produite et distribuée et le risque sanitaire associé à sa consommation par les humains. En effet, la grande majorité des organismes du zooplancton ne constituant pas en eux-mêmes un risque sanitaire, n'étant pas des organismes pathogènes eux-mêmes, ils ont été principalement catégorisés comme un élément esthétique dans la qualité de l'eau potable. Depuis quelques années, les experts commencent à douter de l'innocence parfaite des ces acteurs du monde microbien aquatique, et les amibes ont été les premières accusées d'opérer comme cheval de Troie des microorganismes pathogènes humains, la cible même de toute la désinfection de l'eau potable. On pourrait donc s'inquiéter presque davantage de ces organismes supérieurs, qui circulent librement depuis toujours dans nos systèmes d'eau potable

avec un droit de passage tacitement accepté de tous, que des cibles classique de toutes les persécutions, ces pauvres microorganismes pathogènes constamment surveillés, identifiés à des milles à la ronde et attaqués de toutes parts par nos procédés de désinfection. En réalité, on commence à soupçonner aujourd'hui que ces organismes supérieurs constituent en quelque sorte tout un système de transport en commun en opération depuis toujours à travers nos systèmes d'eau potable, véhiculant ainsi des quantités difficilement estimables de microorganismes pathogènes à travers les procédés de traitement, protégeant ainsi ces microorganismes non seulement de nos caméras de surveillances et de nos radars mais aussi des procédés de désinfection dans une mesure encore une fois jusqu'à ce jour pratiquement indéterminée. Évidemment, ces organismes supérieurs n'opèrent pas simplement et massivement comme d'ordinaires véhicules des microorganismes qu'ils transportent. On trouverait, dans ce système de transport en commun, toutes sortes d'engins, allant de classiques autobus à d'autres machines hautement sophistiquées (voire de la classe de la *BatMobile*?). Et ça ne peut que se compliquer. En effet, à l'échelle des microorganismes, un séjour à l'intérieur d'un véhicule se révèle souvent être une aventure beaucoup plus significative qu'un simple trajet d'un point à un autre, puisqu'il leur pourvoit dans certains cas des conditions favorables à leur multiplication et dans d'autres cas, à leur destruction. Le véhicule n'est effectivement pas qu'un objet en déplacement, mais des interactions plus ou moins complexes avec les passagers méritent également notre attention de scientifiques pour arriver à bien comprendre, et s'il le faut, désamorcer ce réseau de services publics qui nous passe sous le nez dans ce monde microbien étudié pourtant intensivement et avec notre plus grand sérieux. L'enquête à mener reste cependant presque entière; des collectes de données et des analyses poussées seront nécessaires avant de pouvoir intervenir. Cette thèse rassemble différents niveaux d'activités de recherche ayant été menées au cours des 4 dernières années afin d'aborder la question de la présence des organismes supérieurs dans l'eau potable, de leur rôle, leurs implications et leurs responsabilités dans la qualité microbiologique de l'eau, de la nuisance qu'ils peuvent représenter comme obstacle à l'atteinte de nos objectifs de production d'une eau microbiologiquement sûre, et enfin de la nécessité de réviser, de restreindre, plus ou moins drastiquement, le droit de passage de ces organismes supérieurs dans nos systèmes d'eau, ou encore d'apprendre possiblement à contrôler et tirer profit de leur présence, de leurs activités et de leurs capacités pour les mettre au service de nos objectifs de qualité d'eau potable.

INTRODUCTION

1.1 Mise en contexte

L'appellation « organismes supérieurs » désigne un groupe d'organismes naturellement omniprésents dans les eaux naturelles comme dans les systèmes d'eau potable de construction humaine et dont la présence en abondance contrôlée dans l'eau traitée fut historiquement considérée principalement comme un critère esthétique de la qualité de l'eau distribuée. Ce groupe est désigné également comme celui des organismes du zooplancton, qui comprend les protistes (incluant les amibes), des organismes unicellulaires, ainsi que les rotifères et le zooplancton crustacéen (copépodes, cladocères). La problématique associée aux organismes supérieurs en eau potable inclut souvent également les nématodes, qui sont principalement plutôt des espèces benthiques que planctoniques, mais qui sont considérés comme une part permanente de la microfaune aquatique au même titre que les autres organismes du zooplancton. Les nématodes sont souvent rapportés dans la littérature scientifique comme le groupe d'organismes supérieurs le plus abondant à l'effluent des filtres granulaires (Castaldelli et al. 2005) et dans les réseaux de distribution (van Lieverloo et al. 2004).

Suite à des avancées récentes en recherche, la question d'une importance autre qu'esthétique des organismes supérieurs présents dans l'eau potable est soulevéee, considérant les associations possibles de ces organismes à des microorganismes pathogènes et donc leur impact potentiel sur la qualité microbiologique de l'eau et éventuellement en santé publique. En effet, les microorganismes pathogènes, c'est-à-dire la cible première de la désinfection de l'eau potable, développent d'une part de multiples mécanismes de protection face aux conditions environnementales défavorables à leur développement et, d'autre part, font partie d'un complexe réseau trophique au sein duquel ils peuvent être, par exemple, sujets à la prédation par des organismes supérieurs.

Le biofilm est sans doute un des mécanismes de protection des microorganismes pathogènes les plus évolués en termes de complexité de sa structure et de sa résistance aux conditions externes hostiles. La formation d'un biofilm peut être observée, entre autres, lorsque des microorganismes s'attachent à des surfaces biologiques ou inertes, incluant les particules présentes dans l'eau ainsi que la surface de certains organismes supérieurs. Par exemple, l'association à la surface du

zooplancton de la bactérie *Vibrio cholerae*, encore responsable d'une des maladies hydriques les plus sévères dans les pays en développement, est un cas bien documenté soulignant une implication majeure et flagrante des organismes supérieurs dans la qualité microbiologique de l'eau : en effet, des efflorescences de zooplancton dans les eaux côtières ont été associés à des épidémies de choléra au Pérou et au Bangladesh (Huq et al. 1983, Lipp et al. 2003). De plus, l'enlèvement des organismes du zooplancton par une simple filtration sur tissu (~20 μm) a permis de réduire de 2 log (99%) la concentration de *V. cholerae* dans l'eau (Huq et al. 1996) et d'observer une diminution de 48% des cas de choléra dans un village ciblé (Colwell et al. 2003). D'autre part, le développement de la bactérie *Legionella* en association avec les amibes dans les biofilms des réseaux de distribution est une problématique qui a récemment retenu l'attention scientifique en raison de son enjeu en santé publique. En effet, la croissance intracellulaire de *Legionella pneumophila* à l'intérieur d'amibes a été identifiée comme la raison de sa prolifération dans les biofilms des réseaux de distribution (Kuiper et al. 2004). Dans les deux cas, il s'agit de comportements microbiens et d'interactions avec les organismes supérieurs complexes aux niveaux microbiologique et écologique.

La problématique de l'attachement des microorganismes à la surface d'organismes supérieurs n'est pas au cœur de la présente thèse. En effet, la présente étude s'attarde principalement aux cas d'internalisation de microorganismes pathogènes par les organismes supérieurs en eau potable, considérant ainsi les organismes hôtes comme des véhicules des microorganismes internalisés et, jusqu'à un certain point, comme des boucliers face aux procédés de désinfection. Cette perception des organismes supérieurs a souvent été réservée aux amibes, appelées le « cheval de Troie » des microorganismes dans le monde microbien. En effet, suite à l'internalisation de bactéries pathogènes ou opportunistes telles que *Legionella* par les amibes, les bactéries peuvent se multiplier à l'intérieur des vacuoles de l'amibe, de sorte que les amibes, par leur structure résistante, favorisent non seulement la survie, le transport et la protection de bactéries pathogènes humaines, mais également leur multiplication. Dans le cas de *Legionella*, responsable de maladies respiratoires chez les humains, les vacuoles digestives de l'amibe peuvent être expulsées hors de l'amibe en tant que vésicules contenant de nombreuses cellules infectieuses, véhiculées alors en aérosols et engendrant un risque significatif de transmission chez les humains. Il s'agit de loin du cas d'internalisation le plus documenté en termes de santé publique dans l'industrie de l'eau potable. Toutefois, des observations en laboratoires ont révélé

que de nombreuses bactéries pathogènes humaines, incluant des bactéries entéropathogènes telles que *Campylobacter jejuni*, *Escherichia coli* et *Salmonella* sp., peuvent adopter des comportements intracellulaires complexes semblables à ceux de *Legionella* en association avec les amibes, ainsi qu'avec d'autres protozoaires ciliés, comme *Tetrahymena* sp. (Brandl et al. 2005, Snelling et al. 2005). Ainsi, les amibes et d'autres protistes pourraient agir comme vecteurs de microorganismes associés à un risque entérique, et non seulement respiratoire, en eau potable. Ceci n'a toutefois jamais été directement observé en conditions réelles en eau potable, et la prolifération de bactéries associées aux amibes a été principalement rapportée dans les systèmes de distribution de l'eau, à l'extérieur de l'usine de traitement.

Dans le cadre de cette thèse, la problématique de l'internalisation des microorganismes pathogènes par les organismes supérieurs étudiée a été délimitée autour des autres organismes supérieurs dont la présence est fréquemment rapportée en eau potable (i.e. excluant les amibes). Ces autres organismes, tels que les rotifères et les nématodes, par exemple, sont pluricellulaires, contrairement aux amibes. Ainsi, leurs interactions avec les microorganismes sont plutôt du domaine de la prédation, et ne visent pas des mécanismes microbiens intracellulaires comme dans le cas des amibes. C'est pour ces raisons, ainsi qu'en raison de l'attention croissante déjà accordée à la caractérisation du risque microbien associé aux amibes, que ces dernières ont été essentiellement exclues des travaux réalisés au cours de la présente thèse. L'internalisation par les organismes supérieurs telle que cernée dans cette thèse consiste principalement en la persistance de microorganismes à l'intérieur du tube digestif des organismes supérieurs (essentiellement comme des particules inertes), ou encore en leur multiplication dans le cas où, par exemple, la bactérie est pathogène pour l'hôte et peut créer une infection dans son tube digestif. Le premier cas peut être représenté par celui d'(oo)cystes de protozoaires ingérés par exemple par des rotifères, tel qu'observé en laboratoire (Fayer et al. 2000, Trout et al. 2002), alors que la capacité des prédateurs à digérer des structures résistantes telles que la paroi d'(oo)cystes reste à démontrer. Par exemple, aucune excrétion des kystes de *Giardia* ingérés par des rotifères n'a été observée en 20 minutes (Trout et al. 2002), suggérant une persistance et une accumulation possible des kystes à l'intérieur du prédateur. Des oocystes de *Cryptosporidium* ont également été détectés à l'intérieur de rotifères dans des conditions entièrement naturelles, dans le cadre d'échantillonnage d'eaux de lacs (Nowosad et al. 2007), suggérant la possibilité d'une persistance des oocystes ingérés par les rotifères dans des conditions environnementales.

Le second cas, soit celui de l'amplification de microorganismes ingérés par le zooplancton, a été documenté principalement lors d'essais de laboratoires au cours desquels des bactéries pathogènes humaines, telles que *E. coli* O157 :H7, *Salmonella* sp. et *Listeria monocytogenes*, ont été exposées à la prédation par des nématodes, résultant en une infection du tube digestif des prédateurs. De plus, Wolmorans et al. (2005) ont estimé des valeurs moyennes pouvant atteindre 4000 bactéries internalisées par organisme supérieur lors de l'isolation de zooplancton à l'entrée d'un réseau de distribution (en sortie de l'usine de traitement d'eau potable). De telles valeurs laissent supposer une certaine prolifération des bactéries en association avec les organismes supérieurs dans des conditions environnementales.

La problématique décrite dans cette thèse suppose la prédation par le zooplancton dans les filtres granulaires comme principale origine de la présence de microorganismes pathogènes internalisés dans l'eau potable. Cette hypothèse s'appuie entre autre sur les travaux de Locas et al. (2007) qui rapportent une détection récurrente de coliformes totaux internalisés par des nématodes dans un réseau de distribution, comme conséquence de la prédation ayant lieu au niveau de la filtration rapide sur sable à l'usine de traitement d'eau potable en amont. De plus, les organismes supérieurs, principalement les nématodes et les rotifères, sont abondants à la sortie des filtres granulaires (Schreiber et al. 1997, Castaldelli et al. 2005); on peut donc supposer qu'ils transportent une partie des microorganismes internalisés vers la sortie des filtres, dans l'eau filtrée. Enfin, des travaux récents (Hijnen et al. 2007) sur la filtration lente sur sable ont souligné, à titre d'hypothèse, le rôle potentiel des organismes du zooplancton dans l'enlèvement ou la persistance de microorganismes pathogènes résistants tels que les (oo)cystes de protozoaires (*Cryptosporidium*, *Giardia*).

Suite à l'ingestion de microorganismes pathogènes dans les filtres granulaires, le transport et la persistance d'une partie des microorganismes internalisés dans l'eau potable constitue l'axe principal de recherche de cette thèse. La transmission des microorganismes internalisés aux consommateurs d'eau potable suppose en effet la persistance et la survie des microorganismes pathogènes à l'intérieur de leur hôte à l'effluent des filtres granulaires et à travers les barrières subséquentes de désinfection de l'eau potable jusqu'au réseau de distribution. Dans cette thèse, l'étude de la protection des microorganismes internalisés face à la désinfection a été approfondie avec un focus sur la désinfection UV, suite à des travaux récents (Caron et al. 2007) caractérisant l'impact d'autres mécanismes de protection tels que l'attachement aux particules et l'agrégation

des microorganismes face à ce procédé de désinfection physique (optique). La littérature rapporte d'ailleurs plusieurs observations de protection des microorganismes internalisés face aux oxydants chimiques (Chang et al. 1960a, 1960b, Chang et al. 1961, Smerda et al. 1970, Levy et al. 1986, King et al. 1991, Ding et al. 1995, Lupi et al. 1995, Adamo et Gealt 1996, Anderson et al. 2003, Caldwell et al. 2003, Kenney et al. 2004), principalement le chlore, mais aucune recherche n'avait été entreprise sur l'impact de l'internalisation face à la désinfection UV, un procédé de plus en plus répandu dans les usines de traitement d'eau potable des pays industrialisés, entre autres en raison de son efficacité à inactiver le protozoaire *Cryptosporidium*, contre lequel les désinfectants chimiques sont inefficaces et dont les concentrations permises en eau potable sont très faibles.

Un effort a été apporté dans cette thèse afin d'intégrer des travaux sur un procédé de désinfection voisin de la désinfection UV, soit la désinfection solaire, basée sur l'action des radiations UVA naturelles. Ce procédé, concernant à ce jour presque exclusivement des applications dans les communautés en voie de développement, fait l'objet de recherches en cours à travers le monde, sans toutefois qu'on puisse observer une tentative tangible d'y étendre les principales avancées en désinfection UV artificielle. Les travaux expérimentaux réalisés sur l'impact de l'internalisation des microorganismes, en parallèle avec l'attachement et l'agrégation comme mécanismes de protection face à la désinfection UV, ont été étendus à la désinfection UVA à des fins de comparaison. Cette comparaison entre les deux types de désinfection aux rayonnements ultraviolets (UVC artificiels et UVA solaires) constitue donc une innovation au sein de cette thèse.

Enfin, la question de l'importance des organismes supérieurs dans la qualité microbiologique de l'eau potable requiert une analyse poussée des connaissances déjà rapportées dans la littérature et présente une série d'inconnues à combler. Dans une approche d'ingénierie et de gestion, comment répondre à la question « est-ce un risque? » si ce n'est en le comparant avec le risque associé à d'autres problématiques documentées en eau potable. C'est dans cette perspective que l'analyse quantitative du risque microbien (QMRA) a été utilisée comme outil de quantification des diverses variables impliquées dans la chaîne d'événements générant un risque d'infection chez les humains associé à la présence de microorganismes pathogènes internalisés dans l'eau potable.

1.2 Structure de la dissertation

Cette thèse présente d'abord, au chapitre 1, une revue de littérature synthétisant l'état des connaissances scientifiques et des travaux de recherche abordant le rôle des organismes supérieurs dans la protection des microorganismes pathogènes dans l'eau potable (1re publication). Le chapitre 2 présente l'énoncé des objectifs de recherche de la présente thèse et une description de la démarche expérimentale appliquée. Les trois chapitres suivant décrivent des travaux expérimentaux présentés ici dans un ordre calqué sur l'évolution et le transport des microorganismes internalisés à travers le traitement de l'eau potable : le chapitre 3 présente une étude pilote décrivant l'internalisation et le transport d'(oo)cystes de protozoaires à travers la filtration granulaire (2e publication); le chapitre 4 présente des travaux de laboratoire sur la protection de microorganismes internalisés par des nématodes face à la désinfection UV (3e publication); enfin, une comparaison de l'impact des mécanismes de protection des microorganismes face à la désinfection UV et solaire (UVA) est présentée au chapitre 5 (4e publication). Le chapitre 6 présente une analyse de risque (5e publication) intégrant les résultats des travaux précédents dans le développement d'un modèle décrivant l'internalisation d'(oo)cystes de protozoaires dans la filtration granulaire afin d'estimer le risque d'infection associé aux organismes internalisés et véhiculés par les organismes supérieurs dans l'eau consommée par les humains. Au 7e chapitre, une synthèse des travaux ainsi qu'une discussion générale sont exposées. Enfin, les conclusions et recommandations sont énoncées au chapitre 8.

CHAPITRE 1 PUBLICATION #1 - REVUE DE LITTÉRATURE: PROTECTION OF WATERBORNE PATHOGENS BY HIGHER ORGANISMS IN DRINKING WATER: A REVIEW

Ce chapitre présente une revue de littérature publiée dans le *Canadian Journal of Microbiology*. Cette phase de la recherche constitue un point de départ crucial dans la démarche de la présente thèse. En effet, cette revue critique permet d'identifier à la base les principaux chaînons faibles ou manquants dans la littérature portant sur la problématique des organismes supérieurs en eau potable, c'est-à-dire les éléments méconnus qui demandent à être davantage caractérisés afin de pouvoir progresser dans la compréhension du rôle de ces organismes supérieurs dans la qualité microbiologique de l'eau potable produite et distribuée, un sujet novateur au niveau de l'industrie de l'eau potable.

PROTECTION OF WATERBORNE PATHOGENS BY HIGHER ORGANISMS IN DRINKING WATER: A REVIEW

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Abstract: Higher organisms are ubiquitous in surface waters, and some species can proliferate in granular filters of water treatment plants and colonize distribution systems. Meanwhile, some

waterborne pathogens are known to maintain viability inside amoebae or nematodes. The well-documented case of *Legionella*'s replication within amoebae is only one example of a bacterial pathogen that can be amplified inside protozoa's vacuoles, and then benefit from the protection of a resistant structure that favours its transport and persistence through water systems. Yet the role of most zooplankton organisms (rotifers, copepods, cladocerans) in pathogens transmission through drinking water remains poorly understood, since their capacity to digest waterborne pathogens has not well been characterized to date. This review aims at (1) evaluating the scientific observations of diverse associations between superior organisms and pathogenic microorganisms in a drinking water perspective and (2) identifying the missing data which impedes the establishment of cause and effect relationships that would permit to better appreciate the sanitary risk arising from such associations. Additional studies are needed in order to (1) document the occurrence of invertebrate-associated pathogens in relevant field conditions, such as distribution systems, (2) assess the fate of microorganisms ingested by higher organisms in terms of viability and/or infectivity, and (3) study the impact of internalization by zooplankton on pathogens resistance to water disinfection processes, including advanced treatments such as UV disinfection.

Keywords: Drinking water, pathogen vectors, amoebae, nematodes, zooplankton

1.1 Introduction and objectives

The efficiency of disinfection processes in drinking water treatment is influenced to varying degrees by the characteristics of the water (temperature, pH, particle counts, etc.) and the physiologic state of the microorganism being targeted. Rather recently, research has focused on the study of microorganisms in their most common natural form, that is as aggregates or as part of a collective structure known as a biofilm which confers them further resistance to disinfection (Morin et al. 1997, Storey et al. 2004a, Mamane-Gravetz and Linden 2005, Matz and Kjelleberg 2005, Mahmud et al. 2006, Chevrefils et al. 2007).

The protection offered to pathogenic microorganisms located inside superior organisms such as zooplankton organisms (including protozoa) and certain benthic invertebrates is also a natural

protection mechanism used by waterborne pathogens. The protection to disinfectants offered by internalization has not been widely studied. It can be the result of symbiotic or parasitic associations between pathogenic microorganisms and higher organisms (Greub and Raoult 2004). The viability of the pathogenic microorganisms which have been ingested without being digested or biodegraded by their predators can be maintained (Barker and Brown 1994). This resistance to digestion has been reported numerous times in amoebae (Barker and Brown 1994, Winiecka-Krusnell and Linder 1999, Greub and Raoult 2004) and nematodes (Chang et al. 1960, Caldwell et al. 2003, Gibbs et al. 2005) but has not been reported for most zooplankton organisms (rotifers, copepods and cladocerans), even though the study of their predation and grazing activities under diverse conditions has been widely documented.

Higher organisms are ubiquitous in natural and man-made water environments. Planktonic species are commonly found in surface waters, where their occurrence is a function of ecological factors such as seasons, temperature and depth (Pinel-Alloul et al. 2002), and they are part of a complex trophic network in which feeding habits are influenced by various physical and biological factors. (The reader is referred to Pernthaler (2005) for a global and ecological view of the trophic interactions between the numerous members of the aquatic microfauna.) Although for the most part, invertebrates are intercepted and eliminated during sedimentation, some can reproduce inside the plant and liberate eggs and larvae into the distribution system (Levy et al. 1986). Rotifers and nematodes abundantly colonize granular and biological filters, which constitute ideal mediums for the proliferation of benthic invertebrates (Schreiber et al. 1997, Lupi et al. 1994, Castaldelli et al. 2005). They are often released in the filters effluents (Matsumoto et al. 2002) and into the distribution systems. Investigations conducted on several drinking water distribution systems have confirmed the abundance of invertebrates (Chang et al. 1960, van Lieverloo et al. 1998), while amoebae are known to proliferate in the biofilms.

This review discusses the known associations between microorganisms and different groups of higher organisms and presents a critical analysis of research needs, with a specific focus on managing the microbial risk in drinking water. We will present higher organisms in three groups based on ecological characteristics. The zooplankton organisms differ from benthic species by

the fact that they are found in suspension in surface waters and move themselves more or less passively with the currents. As benthic invertebrates, nematodes are usually the object of separate studies, although they are also considered as a permanent part of the aquatic microfauna in surface waters (Lupi et al. 1995). Zooplankton, as studied by limnologists, is typically subdivided in 4 groups: protists (including protozoa and heterotrophic flagellates), rotifers, copepods and cladocerans, the two last groups being known as crustacean zooplankton (Wetzel 2001). In the context of this review, protists will be presented in the first section, as a separate group from the rest of zooplankton organisms: being unicellular organisms, the study of pathogens internalization by protists is of a different nature since it addresses intracellular mechanisms, as opposed to the rest of zooplankton organisms, which are pluricellular and possess a more complex digestive system. From a microbiological point of view, we will see that this difference is of major significance, in an attempt to characterize the fate of internalized microorganisms and the microbial risk that they might confer to drinking water. The second part of this review will focus on rotifers and crustacean zooplankton, and the last part will discuss nematodes as pathogens vectors.

1.2 Protists: The Trojan Horse of Microorganisms

Note: The term "protist" (a unicellular eukaryotic organism) is used in this section as associated with the expression "Trojan Horse" in the literature; however, we are referring here more specifically to protozoa, i.e. protists behaving as animals (with heterotrophic feeding). In fact, according to the information gathered from the literature, plant protists, such as diatoms for example, are not included in the group of organisms referred to as the Trojan Horses of microorganisms, even though certain vegetable protists exhibit bacterivorous behaviour, such as flagellate algae – see Nygaard and Tobiesen (1993) for example.

1.2.1 Amoebae

1.2.1.1 Survival of microorganisms inside amoebae

Protozoa, especially amoebae, have been qualified as the Trojan Horse of the microbial world (Barker and Brown 1994). Amoebae are recognized as being both reservoirs and vehicles of pathogenic microorganisms in the environment, as well as serving as a "crib" (term used by

Greub and Raoult (2004)), i.e. an evolutionary incubator that favours adapting to life within human macrophages, therefore pathogenesis. The reader should refer to the Barker and Brown (1994) article on the impact of predation by protozoa on the survival of pathogenic bacteria in the environment, as well as the article by Greub and Raoult (2004) on amoeba-resistant microorganisms, mainly bacteria, but also viruses.

Free-living amoebae generally have two stages of development: the trophozoite and the cyst (Greub and Raoult 2004). The trophozoite is the active metabolic stage, feeding on bacteria and multiplying by binary fission. Hostile pH conditions, osmotic pressure, temperature, or even unfulfilled nutritional needs of the amoeba can cause its encystment (Greub and Raoult 2004). Cysts generally have two layers, which makes their structure very resistant to most chemical disinfectants, such as chlorine, (Greub and Raoult 2004), and confers them an ability to survive dessication and temperatures between -20ºC and +42ºC (Kahane et al. 2001). When the conditions become favourable once again, there is excystation and a return to active life. Viable bacteria have been observed in trophozoites, as well as in free amoeba cysts (Winiecka-Krusnell and Linder 2001). It is noteworthy to mention that cyst formation is a mechanism common to many protozoa, and is not exclusive to amoebae.

In water, free-living amoebae often live in biofilm, as well as in water-earth, water-air, and water-plant interfaces, since feeding in most species occurs in association with surfaces and particulate matter suspended in water (Greub and Raoult 2004). Amoebae, like other protozoa, feed mainly on bacteria, many of which are able to survive following ingestion by amoebae. The most well known example in the field of drinking water is by no doubt the case of *Legionella pneumophila*, responsible for numerous cases of respiratory illness throughout the world. Survival and transmission of *L. pneumophila* to humans is strongly linked to the presence of amoebae in water, since free-living amoebae favour the multiplication of *Legionella pneumophila* in aquatic biofilms and the transport of the bacteria (Greub and Raoult 2004). Intracellular growth inside of amoebae was even demonstrated to be most likely the only way for *L. pneumophila* to proliferate within aquatic biofilm on plasticized polyvinyl chloride in a batch system (Kuiper et al. 2004).

Microorganisms that resist ingestion by amoebae and other protozoa can be divided into three groups: those that multiply and cause cellular lysis in amoebae, such as *Legionella* and *Listeria* bacteria, those that multiply within the amoeba without causing cellular lysis, such as *Vibrio cholerae*, and those that survive within the amoeba without multiplying, such as certain coliforms and mycobacteria (Barker and Brown 1994). Furthermore, Greub and Raoult (2004) have identified a group of bacteria called "LLAP" (*Legionella*-like amoebal pathogens), including bacteria which, like *Legionella*, are able to cause lysis in the amoeba that carries them. This group of bacteria is attracting more and more attention due to public health concerns.

Thus, besides predator-prey relationships, cases of parasitism and even of endosymbiosis can be observed in certain bacteria or viruses that survive following their ingestion by the amoeba and avoid being digested. This endosymbiosis can take place initially as a survival strategy adopted by a microorganism facing hostile conditions or physical variations in its environment (Winiecka-Krusnell and Linder 2001). This type of association is not only of considerable importance for the stabilization of infectious agents in the environment, but can also increase the potential virulence of bacteria that can evolve to become highly adapted to intracellular growth (Barker and Brown 1994). In fact, certain bacteria, including *Legionella* sp., *Listeria monocytogenes*, or *Mycobacterium avium*, have adapted to living inside human macrophages following exposure to environmental predators such as free-living amoebae (Greub and Raoult 2004). For example, in both macrophages and amoebae, *Legionella*'s survival is characterized by the absence of phagosome-lysosome fusion, which somehow impedes the cell's digestion of the bacterium, and in both cases (macrophages and amoebae), *Legionella* leads to cellular lysis (Greub and Raoult 2004). Hence, it is probable that *Legionella*, as some other intracellular pathogens, has evolved thanks to its association with protozoa in the natural environment in such a way that it acquired the ability to infect humans or other animals. However, despite bacteria survival within protozoa often being associated with their pathogenesis, it is noteworthy that protozoa, including amoebae, can also serve as reservoirs of environmental bacteria, such as non pathogenic coliforms (King et al. 1988), and can protect them from hostile environmental conditions or chlorination.

Literature contains many reports of laboratory experiments in which human pathogenic bacteria were maintained in coculture with various species of *Acanthamoebae*. In most of these studies, bacteria were observed to maintain their viability and multiply inside the amoeba's feeding vacuoles. For instance, *Helicobacter pylori* was found to preserve its viability and proliferate inside of *Acanthamoebae castellanii* for up to 8 weeks in coculture (Winiecka-Krusnell et al. 2002). Intact and metabolically active bacteria have been observed in amoebae's vacuoles and a 2-log increase in *H. pylori* bacterial count was observed after 7 days of coculture with *A. castellanii*. Interestingly, when in coculture for one week with various species of *Listeria*, *A. castellanii* was observed to undergo cell lysis and release viable bacteria of *L. monocytogenes* and *L. seeligeri*, two haemolytic species, while *L. innocua*, a non pathogenic species, was not freed from the amoeba (Ly and Müller 1990b). In similar experiments, amoeba encystment occurred by day 8 of incubation of *L. monocytogenes* with *A. castellanii* (Ly and Müller 1990a). After 34 days of coculture, almost all of the amoebae were found to be in cyst form, inside of which *L. monocytogenes* had lost its viability. Three serotypes of *Salmonella enterica* (serovar Dublin, Enteritidis, and Typhimurium) were shown to reside and replicate within intracellular vacuoles of *A. rhysodes* (Tezcan-Merdol et al. 2004). A prolonged incubation of the *Salmonella* and *Acanthamoeba* coculture resulted in a gradual change in the host cells morphology until they eventually disappeared. *Simkania negevensis*, associated with respiratory illnesses in humans, was reported to infect *A. polyphaga*, survive and reproduce within trophozoite vacuoles, as within human cell cultures (Kahane et al. 2001). Furthermore, exposure to hostile conditions caused amoeba encystment, and a certain competition was then observed between the amoeba and the bacteria for survival, resulting in three possible behaviours: cysts containing both normal cytoplasm and *S. negevensis*, cysts containing bacteria but without cytoplasm, or finally, the bacteria were found located between the two cyst walls. After 79 days at 4º C, the *S. negevensis* bacteria caught inside the cysts had preserved 56% of their infectivity, while free bacteria (the control sample) had not survived 12 days of exposure to the same temperature. In addition, a small proportion of the bacteria (0.3% of the initial infectivity) had survived as long as 21 weeks (148 days) inside the cysts at room temperature. In the drinking water industry, it is most relevant to draw specific attention to those bacteria that showed an ability to survive and retain infectivity inside the amoebal cysts, since (oo)cysts are resistant enough to successfully penetrate and persist through the various steps of water treatment plants.

The ability of 26 species of water-related mycobacteria to survive inside trophozoites and cysts of *A. polyphaga* was assessed in a recent study (Adékambi et al. 2006). All species studied showed the ability to penetrate into trophozoites and cysts, where they can survive more than 5 or 15 days, respectively. *Campylobacter jejuni* was also shown to infect *A. polyphaga* in vitro at different temperatures typically found in natural waters (Axelsson-Olsson et al. 2005). In fact, aggregation of a great number of motile and active bacteria was observed within vacuoles of the amoeba. The spontaneous rupture of the amoeba allowed the detection of *C. jejuni* by microscopy and by culture. Further studies are required to verify whether (1) amoeba infection by *C. jejuni* can occur naturally in the environment and (2) if bacteria that survive in the amoeba are able to infect a vertebrate host (Axelsson-Olsson et al. 2005). Investigation on these aspects was actually found to be lacking from most studies about intracellular replication of bacterial pathogens in amoebae. In fact, these studies are crucial in understanding the ecology of pathogens intracellular survival inside protozoan hosts. They also provide qualitative information on the potential microbial risk. However, very few studies have reported the occurrence of infected amoebae in natural or man-made environments. This information is needed in order to better discriminate which pathogens are really associated to an increased risk of transmission to humans when amoebae are present in drinking water treatment or distribution systems. Furthermore, with respect to risk quantification, the laboratory experiments are not sufficient, since coculture assays most likely overestimate the number of bacterial pathogens associated to amoebae as compared to what would be found under field conditions.

Even though the role of amoebae as bacterial reservoirs has long been known, Robotham was the first researcher, in 1980, to shed light on the role of amoebae as vector of *Legionella*, which would be favourable to their propagation in drinking water systems as well as in humans (Greub and Raoult 2004). Amoebae's digestive vacuoles containing bacterial cells following ingestion can be expulsed from the protozoa, which often precedes encystment (Berk et al. 1998). Expelled vacuoles are called vesicles (Brandl et al. 2005). In the case of *Legionella*, a single vesicle can contain up to 10^4 bacteria according to the calculations of Robotham (1986), while the infectious dose for humans is assumed to be very low. When *A. polyphaga* and *A. castellanii*,

rejected vesicles that contained viable *L. pneumophila* cells, it was found that more than 90% of these vesicles were small enough to be inhaled, i.e. less than 5 μm in diameter (Berk et al. 1998), which supports the hypothesis that humans contaminated with *Legionella* would possibly have inhaled a vesicle that was filled with bacteria rather than free bacteria (Greub and Raoult 2004). Moreover, these vesicles contained viable bacteria despite a 24-hour exposure to biocides used in cooling towers (Berk et al. 1998). The bacteria aggregated together within these vesicles and did not disperse despite freezing and thawing treatments (-70^{o}C and 35^{o}C) and ultrasound. The vesicles remained intact following these treatments, contrary to the trophozoites that were completely destroyed under the same conditions (Berk et al. 1998). *Acanthamoeba polyphaga* was shown to produce up to 25 vesicles in 24 hours under certain conditions, following *Legionella pneumophila* ingestion (Berk et al. 1998). These vesicles were found to be free in solution. Considering that amoebae, in the trophozoite or cyst stages, usually adhere strongly to a physical substrate, this observation suggests that these vesicles would further facilitate the bacterial dissemination as aerosols rather than the amoebae themselves (Berk et al. 1998).

In summary, these studies reveal many interesting points regarding amoebae as a risk factor in water systems: amoebae (1) favour the replication of human pathogens inside their digestive vacuoles, (2) increase the survival time and resistance to harsh conditions of those protected pathogens, (3) can enhance the potential virulence of those pathogens by favouring adaptation to intracellular survival and growth, (4) favour the transport of pathogens inside vesicles, which are resistant to extreme temperatures and are found free in solutions, (5) favour the transmission of pathogenic bacteria to human by inhalation of vesicles, considering that a single vesicle can contain the human infectious dose. In terms of risk of transmission to humans, the study of bacteria's survival within amoebal cysts and vesicles, considering their higher resistance to harsh external conditions, is more important than survival within trophozoites, and therefore should deserve more attention in future research work.

1.2.1.2 Protection of ingested microorganisms by amoebae against water treatment

After having been shown to ingest various species of *Legionella*, two species of *Acanthamoeba A. castellanii* and IA, of clinical and environmental origin respectively, were shown to increase by one to two logs the survival of intracellular bacteria *L. erythra* and *L. pneumophila* against

thermal treatment (with temperatures varying from 40 to 80 ^{o}C), when compared to planktonic bacteria (the control sample) (Storey et al. 2004b). *L. erythra* and *L. pneumophila* were found inside amoebae's vacuoles. However, both species of *Legionella* were more easily disinfected by free and combined chlorine when in contact with *Acanthamoeba*, compared to their planktonic state. This surprising result is perhaps due to *Legionella*'s ability to adopt a superior state of resistance in reaction to hostile conditions, just like other non spore-forming bacteria, which would be the case with planktonic *Legionella*, while the opposite situation could occur in protected bacteria which are in an intensified metabolic state within the amoeba, which could render them more vulnerable to oxidation (Storey et al. 2004b). These observations call for more intense investigation, since they contradict the usual conclusions reported in literature (King et al. 1988, Barker and Brown 1994).

Besides *Legionella*, pathogenic bacteria such as *Salmonella typhimurium, Yersinia enterocolitica, Shigella sonnei,* and *Campylobacter jejuni*, as well as environmental coliforms including *Escherichia coli*, have the ability to survive following ingestion by *Acanthamoeba castellanii*. This association was shown to increase by 30- to 120-fold the resistance of all of these bacteria to free chlorine residual, surviving exposure to chlorine doses well above the necessary dose corresponding to a 2-log-inactivation of free-living cells of the same bacteria (King et al. 1988). One to three bacteria per vacuole were found in most of the amoebae following coculture with each of the bacteria. Furthermore, various species of water-related mycobacteria maintained in coculture with *A. polyphaga* were shown to survive a 24-hour exposure to 15 mg/L of free chlorine while protected inside of cysts (Adékambi et al. 2006).

In summary, certain types of interaction between pathogenic bacteria and amoebae, offer significant protection to intracellular bacteria against chemical disinfectants, besides favouring their multiplication and transport and increasing their virulence potential. As these interactions have been well documented, additional work is needed in order to fulfill the lack of quantitative information which impedes a rigorous quantitative assessment of the risk factors associated with amoebae harbouring pathogens in drinking water systems. An improved risk characterization

could potentially influence the disinfection strategies adopted in some drinking water treatment systems. This issue will be further discussed later in the text.

1.2.2 Ciliated Protozoa

1.2.2.1 Survival of microorganisms inside ciliated protozoa

Apart from amoebae, other protists are known to favour the survival of pathogenic microorganisms in the environment, those being mainly the ciliated protozoa of the *Tetrahymena* or *Cyclidium* genus. The relationships are often comparable to those observed in amoebae. *Tetrahymena pyriformis*, an aquatic and bacterivorous ciliated protozoan that feeds by filtration, was found to be widely used in coculture studies. Thus, *L. pneumophila* and *L. monocytogenes* ingested by *Tetrahymena* will multiply within the host and cause cellular lysis (Barker and Brown 1994). After 8 to 15 days of coculture, *T. pyriformis* lysis lead to viable *L. monocytogenes* being freed (Ly and Müller 1990a). The same phenomenon was observed in *L. seeligeri*, while the contrary occurred when very few host cells underwent lysis by the non pathogenic and non haemolytic *L. innocua* (Ly and Müller 1990b), despite *T. pyriformis* being just as densely colonized (6 to 9 x 10^3 bacteria per cell) as they were with the two other species of *Listeria*. A relatively constant coexistence of *L. innocua* and *T. pyriformis* populations was observed for 5 weeks, with the majority being intracellular bacteria. Conversely, in the presence of *L. monocytogenes* and *L. seeligeri*, *T. pyriformis* completely disappeared after about 10 days of incubation, which was followed by the presence of a free *Listeria* population, which declined up until its disappearance at the end of 5 weeks (Ly and Müller 1990b). Those results suggest that *L. monocytogenes* and *L. seeligeri* would parasitize *T. pyriformis* whereas *L. innocua* would seem to simply resist digestion by the ciliate without causing its lysis.

Campylobacter was shown to survive inside *T. pyriformis* and *Acanthamoeba castellanii*. When incubated in the presence of both protozoa, the survival of *C. jejuni* was increased by 36 hours compared to planktonic bacteria (Snelling et al. 2005). However, the presence of *T. pyriformis* did not significantly delay the decline in viability of *Campylobacter coli*, while coculture with *A. castellanii* delayed it by about 24 hours, therefore suggesting that the relationship between *Campylobacter* and protozoa is species-specific.

A comparison of the ingestion of *Salmonella enterica* serovar Thompson and *Listeria monocytogenes* by *Tetrahymena* lead to the liberation of numerous vesicles containing viable *Salmonella* cells, while ingestion of *Listeria* by the protist resulted in their digestion. The expulsion of vesicles containing *Listeria* was infrequent (Brandl et al. 2005). Up to 50 *Salmonella* cells per vesicle expelled by *Tetrahymena* were counted, this number increasing with the initial ratio of bacteria to protozoa cells in coculture. The difference in the ways these two bacteria interact with the protozoa was probably not simply due to the difference in the nature of their cell walls (Brandl et al. 2005), since *Enterococcus avium*, a gram-positive bacterium, just like *L. monocytogenes*, resisted being digested by *Tetrahymena*. The authors suggest that serovar Thompson is able to alter the normal sequence of events linked to digestion in the *Tetrahymena* digestive vacuoles by stopping the fusion of the phagosome to the lysosome, for example, in the same way as *Legionella* resists digestion inside amoebae and human macrophages.

Various ciliated protozoa were shown, in laboratory conditions, to ingest *Cryptosporidium* oocysts (Stott et al. 2001). Ingestion rates were observed to (1) vary in time, (2) generally increase according to prey concentration and (3) vary significantly from one species of ciliated protozoa to another, the most efficient protozoa studied being *Paramecium caudatum*, which could ingest up to 170 oocysts per hour. Furthermore, a relationship between the average number of oocysts ingested and the average size of the protozoa was proposed. Even though the fate of ingested oocysts was not determined, and despite the short test time of protozoa feeding (1h), the authors suggested the possibility of some ingested oocysts being digested or excreted by their predators. In fact, immunofluorescence assays made it possible to detect fragmented oocyst cell walls inside of protozoa digestive vacuoles and certain *Stylonychia mytilus* have excreted particle debris containing many oocysts whose viability has not been determined. The authors are aware that their laboratory results are probably not representative of natural phenomena, since ingestion of oocysts by protozoa in the environment can depend on their feeding habits, population diversity and density, exposure time, and oocysts distribution. It was observed that once the ciliate *Paramecium caudatum* is exposed to 90 or 9,000 *Cryptosporidium* oocysts for 20 minutes, individual protozoa ingests 1.38 or 26.7 oocysts on average, respectively (Stott et al. 2003).

After one hour of exposure to the highest oocyst level, the number of oocysts ingested by *P. caudatum* was repeatedly found to be higher than the human infectious dose of 30 oocysts.

Contrary to natural water studies carried out by ecologists, the studies that describe the ingestion ability of zooplankton organisms under artificial conditions have the advantage of supplying precious information on ingestion of waterborne pathogens, which are the main focus of researchers in the field of drinking water treatment, whose priority is to reduce health risks. However, it is important to note that the experimental conditions are often very different from the conditions prevailing in natural aquatic environments. More specifically, the disproportion of microorganism densities in these laboratory tests compared to natural concentrations in aquatic ecosystems is evident. In addition, none of the reported studies using *Cryptosporidium* as a food source for higher organisms have investigated the viability and infectivity of the oocysts after their ingestion or excretion. Future studies should include such an investigation, since this aspect is determining in the assessment of the potentially associated health risk.

1.2.2.2 Protection of ingested microorganisms by ciliated protozoa against water treatment

Just like Acanthamoeba, Tetrahymena pyriformis ingests coliform bacteria as well as the pathogenic bacteria Salmonella typhimurium, Yersinia enterocolitica, Shigella sonnei, Legionella gormanii and Campylobacter jejuni, which survive within the cell where they are protected against chlorination (King et al. 1988). The resistance of all these pathogenic bacteria against chlorination was observed to be more than 50 times higher when ingested by T. pyriformis. For the sake of comparison, a freshwater environmental protozoan of the genus Cyclidium was isolated and the necessary contact time for inactivation of 2 logs of E. coli by chlorine was even greater when in contact with Cyclidium than with T. pyriformis. It was also found that internalization of Campylobacter by T. pyriformis and Acanthamoeba castellanii significantly increases its resistance to a chemical disinfectant widely used in the poultry industry (Snelling et al. 2005). Coculture of Salmonella enterica serovar Thompson with Tetrahymena showed that expelled vesicles containing viable bacteria offered a significant protection for Salmonella against a free chlorine treatment of 4.2 mg·min/L, as the average proportion of bacteria surviving

the treatment when located inside a vesicle was 4.6-fold higher compared to free bacteria (Brandl et al. 2005).

In summary, additional studies are needed to quantify the increase of pathogens resistance to disinfection, including UV treatment, as most studies relied on free chlorine. These studies can be performed in artificial conditions in research laboratories. However, the challenge resides in a proper assessment of the natural occurrence of this phenomenon in surface waters and in distribution systems. This highlights the importance of having ecologists and limnologists collaborating with water engineers on such an issue, so that pathogenic microorganisms receive specific attention when characterizing (quantitatively) trophic relationships in natural aquatic environments.

1.3 Rotifers and Crustaceans

1.3.1 Survival of microorganisms inside zooplankton

A few rare studies have attempted to characterize the survival of microorganisms ingested by zooplankton organisms other than protists. Even less frequent are such studies performed in natural conditions. Nevertheless, the use of rotifers was suggested as a *Cryptosporidium* oocyst detection tool in Polish lake waters and made it possible to detect viable oocysts contained within rotifers in each of the three lakes sampled (Nowosad et al. 2007). This is probably the first record of the ability of *Cryptosporidium* oocysts to survive inside of zooplankton organisms in natural waters. The oocysts' infectivity has not been verified, however. We note that, in natural conditions, it is common to find densities of 200 to 300 rotifers per litre, and occasionally up to 1,000 individuals per litre (Wetzel 2001).

Most studies found in literature about zooplankton grazing in natural conditions characterize their grazing rates on various species in the microbial community, or the impact of a zooplankton species population on other planktonic organism concentrations. However, these studies usually do not provide any information on zooplankton grazing on waterborne pathogens. That is why laboratory experiments are necessary in order to understand the significance of higher organisms in the context of drinking water. For instance, laboratory experiments were conducted to

investigate the fate of ingested *Cryptosporidium parvum* oocysts and *Giardia lamblia* cysts by rotifers and daphnia. Under artificial conditions, 20,000 *C. parvum* oocysts were exposed to populations of 10 to 20 individuals of six rotifer species (Fayer et al. 2000), which were all observed to ingest oocysts. Up to 25 oocysts were found inside of *Philodina* rotifers, the majority containing about 15 oocysts. *Euchlanis triquetra* and *Epiphanes brachionus* rotifers were seen as excreting aggregates containing up to 8 oocysts, about 15 minutes after the beginning of exposure of the rotifers to oocysts. (It is known to take between 3 and 20 minutes, according to the rotifer species and the environmental conditions, for a particle ingested by a rotifer to move along its entire digestive tract (Wetzel 2001)). The other rotifer species seemed to keep the oocysts internally for the entire duration of the microscopic observation. However, there is no report of oocysts being degraded, digested, or inactivated inside rotifers following ingestion, as it is not known whether or not rotifers have any enzymes that can digest the proteins forming oocysts cell wall (Fayer et al. 2000). Rotifers enzymes identified to date mainly digest carbohydrate substrates. This study consists in artificially exposing rotifers to *C. parvum* oocysts. The authors did not determine whether or not rotifers ingest oocysts in nature, which, however, has been recently shown by Nowosad et al. (2007). Assays by Stott et al. (2003) also showed that following a 2-hour exposure, rotifers ingested an average of 1.6 oocysts per individual, with the maximum observed being 7 oocysts. It is important to note that again, in this study, predators were exposed to oocyst concentrations in the order of 10^4 to 10^6/ml, while typical concentrations in drinking water are less than 0.001 oocyst/ml (Brookes et al. 2004). Oocysts were also seen in rotifers fecal matter after 145 minutes, but oocyst viability following ingestion was not determined (Stott et al. 2003). No oocyst accumulation inside of predators was observed during the assay period. According to the authors, the impact of higher organism predation could potentially reduce the presence of *Cryptosporidium* oocysts in the environment, but zooplankton organisms could also become reservoirs and vectors, therefore favouring transmission of *Cryptosporidium*. When exposing seven species of rotifers to high concentrations of *Giardia* cysts, five species ingested cysts in variable quantities. The cysts remained within their bodies for the entire observation period of 20 minutes (Trout et al. 2002). In general, rotifers ingested cysts in smaller quantities than those reported for *Cryptosporidium parvum* oocysts, which the authors hypothetically attribute to *Giardia* cysts being 3 to 4 times larger than oocysts, and their surface possibly having different characteristics. No species of

rotifer seems to have excreted *Giardia* cysts. It is not known whether or not the cysts were digested. These studies being done in artificial conditions, the authors emphasize that it has not been determined whether or not rotifers would ingest *Giardia* cysts in a natural environment. More in-depth studies would be required to (1) quantify the probability that rotifers ingest *Cryptosporidium* or *Giardia* (oo)cysts in natural conditions and (2) determine the impact of ingestion (and defecation) on the viability and infectivity of (oo)cysts.

With regard to crustaceans, studies concerning the survival of ingested microorganisms seem to be limited mainly to one single host, which are cladocerans of the genus *Daphnia*. A recent study by Connelly et al. (2007) describes the effect of grazing by *Daphnia pulicaria* on the density, viability and infectivity of *C. parvum* oocysts and *Giardia lamblia* cysts under artificial conditions. *C. parvum* oocysts outer wall were not disrupted, or slightly in some rare occasions, following ingestion and excretion by *D. pulicaria*, whereas *G. lamblia* cysts were highly disrupted, probably due to their larger size. The authors suggest that repeated ingestion of the (oo)cysts might have occurred during the 24-h grazing period, considering the high concentrations of pathogens and grazers. Distinction was not possible between (oo)cysts that would have been ingested multiple times from those that were never ingested by the daphnia. It was suggested that that repeated ingestion and excretion of *G. lamblia* cysts might explain the mechanical damage to the cysts wall, which might have interfered with the measurements of excystation following grazing. In fact, grazing was shown to significantly decrease the viability of *Giardia* cysts (based on standard DAPI-PI vital dye staining techniques) but to increase excystation, which could be attributed to the mechanical disruption of the cysts due to digestion, leading to the release of trophozoites. In the case of *C. parvum* oocysts, they report a significant decrease (87%) in the mean oocysts infectivity due to grazing by *D. pulicaria* in their assay conditions, as measured by in vitro cell culture assays. Although the conditions prevailing in natural systems must be considered when evaluating the impact of zooplankton grazers on human pathogens in water, the authors conclude that *D. pulicaria* can significantly decrease the concentration of infectious (oo)cysts in natural surface waters.

As for the ingestion of bacteria, after exposing *Daphnia carinata* to *Campylobacter jejuni* for 72 hours, an average of 33 bacteria was found in association with the surface or the inside of each daphnia, and a grazing rate of 1.75 ml/individual/h was calculated (Schallenberg et al. 2005). This value coincides with the typical values found in literature for natural conditions, since daphnia are known to graze efficiently in lakes and ponds, at typical rates varying between 0.1 and 2.8 ml/ind./h. Daphnia density for the assays was 40 daphnias/L, which is representative of natural conditions, knowing that the occurrence of daphnia is most often greater than 30 ind./L and can exceed 100 ind./L (Schallenberg et al. 2005), while the initial *C. jejuni* density was between 1.4×10^6 and 1.0×10^3/ml, typical of wastewater concentrations. Following 72 hours of exposure, *D. carinata* had reduced the *C. jejuni* population by 99% (2 logs) compared to the control (absence of *D. carinata*). Thus, the authors concluded that *C. jejuni* ingestion by *D. carinata* caused the death of the bacteria and they put forth the hypothesis that daphnia, when present in a high enough density, could reduce the concentration of this pathogenic microorganism in aquatic ecosystems.

Other laboratory assays were carried out in order to verify bacteria's ability to survive digestion by Daphnia *ambigua*, a well known bacterivorous cladoceran that had previously been found to be abundant in natural lake water (King et al. 1991). *Staphylococcus* cocci-shaped bacteria, *Alcaligenes* and *Pseudomonas* died following *Daphnia* ingestion, while *Corynebacterium* rod-shaped bacteria survived digestion. The authors suggest that rod-shaped bacteria survive digestion by *D. ambigua* while the coccoidal do not, but they add that a certain allelopathy (or amensalism) could take place in the *D. ambigua* digestive tract, i.e. a type of microbial competition that would be the cause of *Staphylococcus*'s death rather than the digestion by its host. In fact, they observed that *Staphylococcus* survived 18 hours longer after being ingested by *D. ambigua* in the absence of *Corynebacteria*, even though it was digested after 19 hours. Both *Staphylococcus* and *Corynebacteria* are gram-positive bacteria. The authors put forth the hypothesis that zooplankton could possibly select gram-positive bacteria to feed upon, while it has already been seen that protozoa, whose feeding is characterized by a passive mechanical selectivity (Wetzel 2001), ingest gram-positive bacteria at lower rates than gram-negative bacteria (Pernthaler 2005).

1.3.2 Protection of ingested microoorganisms by zooplankton against water treatment

On-site studies were conducted to isolate and identify bacteria associated with zooplankton in Lake Oglethorpe, a stratified eutrophic and shallow lake in the state of Georgia, USA (King et al. 1991). Water samples containing zooplankton were harvested and chlorinated (10 mg/L NaOCl for 5 min) so as to eliminate planktonic bacteria and conserve only bacteria associated with zooplankton. The ingested bacteria were then freed by putting the zooplankton samples through ultrasound. Bacteria that were cultured from both raw water samples and treated zooplankton samples were assumed to survive zooplankton digestion. Bacteria found in contact with zooplankton, and particularly within the digestive tract, were protected from chlorination, since they could be cultivated following freeing by ultrasound, meanwhile no non-spore forming bacteria had been reported to date to be able to survive a dose of free chlorine such as the one applied to zooplankton samples. It was also shown that bacteria such as the coliforms *Enterobacter cloacae* and *Klebsiella pneumoniae* isolated from drinking water, and *Salmonella livingstone*, isolated from wastewater, could be protected from chlorine and monochloramine disinfection and remain viable inside of the digestive tract of the crustacean amphipod *Hyalella azteca* used as a model invertebrate for these assays (Levy et al. 1986).

From this section, it is important to emphasize the fact that pathogenic protozoa such as *Cryptosporidium* and *Giardia*, which exhibit low infectious dose, could be located inside a zooplankton organism in natural waters. Therefore, it is necessary to assess to what degree this occurrence actually translates into an increased risk of infection, especially in the case of, unfiltered waters treated with UV radiation and distributed to consumers. Furthermore, there is evidence that some bacteria can survive within zooplankton organisms, whereas others are mostly digested and biodegraded. Therefore, we stress the need to better understand the ecology of pathogens in natural aquatic environments, in order to characterize the fate of pathogens ingested by zooplankton. Surprisingly, there is barely any information in the literature to this date about the protection effect of rotifers and crustacean zooplankton against common water treatment processes.

1.3.3 Bacterial Colonization of the Exoskeleton's Surface of Zooplankton Organisms

While there is a lack of information on zooplankton harboring pathogenic microorganisms in water, attachment to planktonic animals is more documented as a vectoring mode for waterborne pathogens. Certain bacteria can attach themselves to the surface of zooplankton organisms where they find a microhabitat that makes it possible for them to persist longer in the environment. To date, the most studied and relevant case in the field of water is that of *Vibrio cholerae*, the bacterium responsible for cholera. For an exhaustive review of *Vibrio cholerae* ecology and microbiology, the readers are invited to refer to Cottingham et al.(2003).

In various species of marine copepods, there is an intestinal flora that includes many types of heterotrophic bacteria and that is dominated by *Vibrio* (Sochard et al. 1979). Copepod defecation was observed as a means of bacterial dispersion in the water and marine sediments, since bacterial counts during copepod digestive tract dissection were lower after defecation. Other bacteria, such as *Pseudomonas* and *Cytophaga* were found to be associated with copepods, without being able to specifically associate them with the digestive tract, however. Considering the sum of the bacteria attached to the surface of copepods and those found in their intestinal flora, it was suggested that there are a greater number of bacteria actually associated with copepods rather than free in the water column. Researchers have therefore studied the association of bacteria with zooplankton organisms, the most documented case being that of *Vibrio cholerae* attachment particularly to the surface of copepods, given its epidemiological importance. Some have studied the relationship between *V. cholerae* and zooplankton in estuary zones (Huq et al. 1983, Tamplin et al. 1990, Huq et al. 1996, Chiavelli et al. 2001, Colwell et al. 2003, Lipp et al. 2003, Cottingham et al. 2003, Huq et al. 2005, Kirn et al. 2005, Alam et al. 2006), but in freshwater as well (Sarkar et al. 1983), and have suggested a link between episodes of zooplankton abundance and the occurrence of cholera epidemics in certain developing countries, such as Peru and Bangladesh. Up to the beginning of the 1980's, the detection of *V. cholerae* was linked to temporally sporadic events that coincided with epidemics in geographical areas where this bacterium is endemic. However, the existence of a non detectable state of bacteria, the "viable but not culturable" state, was discovered to be the cause of the latent periods

during which *V. cholerae* was not detected, even though it was present in natural waters between two epidemics (Xu et al. 1982, Binsztein et al. 2004). This behaviour in *V. cholerae*, as well as its attachment to zooplanktonic organisms, is crucial in understanding its ecology and cholera prevention: for example, Xu et al. (1982) put forth the hypothesis that these viable but not culturable *V. cholerae* cells could survive attached to copepods, then reproduce once optimal conditions arose. Huq et al. (1983) observed that the presence of live copepods increased the survival time of *V. cholerae* in water. The relationship between *V. cholerae* and planktonic copepods could explain the seasonal cholera epidemics, for example in Bangladesh, where an epidemic begins almost every year in September or October (Huq et al. 1983), shortly following the annual zooplankton bloom in coastal waters. Sanitary concerns are therefore linked to *V. cholerae* colonization of the surface of copepods, and other chitinous organisms, considering that a single copepod could support a *V. cholerae* population on its surface sufficient to cause cholera in a human (Huq et al. 1983). Furthermore, bacteria were found to colonize the oral area and egg sac of copepods, where cell division was observed, indicating bacterial multiplication (Huq et al. 1983). The concentrated bacterial adhesion near the crustacean's mouth would suggest that it could serve as food, which could lead to *V. cholerae* being dispersed into the aquatic environment if the bacteria happen to multiply within its host's digestive tract before being excreted in the copepod's fecal matter.

V. cholerae also attaches to many species of cladocerans and rotifers (Tamplin et al. 1990) and to certain species of phytoplankton. Therefore, a simple filtration method on tissue was proposed to extract the bacteria that was attached to plankton in raw waters in developing countries (Huq et al. 1996, Colwell et al. 2003). The method was tested on different strains of *V. cholerae* O1 and O139 originating from many different geographical areas, namely Bangladesh, Brazil, India, and Mexico. The results showed a 99% (2 logs) removal of *V. cholerae* (Huq et al. 1996). A field study in Bangladesh showed effective removal of particles greater than 20 μm and a 48% reduction in cholera cases in the villages that used this filtration method, compared to the control villages (Colwell et al. 2003).

The nature of the relationship between *Vibrio cholerae* and copepods has long been unexplained, except for the fact that the planktonic animal's chitinous shell was an adequate environment for the survival and growth of the bacteria. Bacteria of the genus *Vibrio* associated with zooplankton were reported to play an important role in chitin mineralization by bonding to the chitin and using it as an exclusive source of carbon and nitrogen (Heidelberg et al. 2002). Also, the presence of pili on bacterial cells, as is the case with *Vibrio cholerae*, is often associated with the ability to colonize surfaces. Further to being associated with zooplankton, *V. cholerae* is also found in the aggregates of natural biofilms that are either floating or attached to debris (Alam et al. 2006), which can also provide a favourable environment for the persistence and proliferation of *V. cholerae* O1. The formation of biofilm by *V. cholerae* greatly increased their resistance to predation by protozoa compared to planktonic bacteria, notably because bacterial density in biofilms allows, according to the *quorum sensing* principle, the production of an exopolysaccharide that inhibits protozoa grazing activity (Matz et al. 2005).

In an attempt to characterize the health risk associated to zooplankton's surface attachment, it is important to note that *V. cholerae* is not the only pathogenic bacterium to adopt such a strategy in aquatic environments. *Helicobacter pylori*, responsible for gastric ulcers in humans, can also attach itself to the surface of cladocerans and copepods, which would suggest that planktonic organisms provide a means of possible transmission of this bacterium to humans (Cellini et al. 2004). The significance of pathogenic bacteria's attachment to zooplankton's surface has never been quantified as a risk factor in microbial risk assessment associated with drinking water. However, the simple filtration technique recommended in developing countries provides evidence that the removal of zooplankton organisms in drinking water can lower the risk of cholera infection in regions where *V. cholerae* is endemic. This reveals the potential sanitary significance of such associations with higher organisms and the relevance of studying them in drinking water. In addition, it is not understood at this time whether or not biofilms forming on biological surfaces such as zooplankton exoskeleton do provide a significant increase in resistance to disinfection treatments, including chemical disinfection and UV. Further work is therefore necessary to answer those questions.

1.4 Nematodes

Nematodes are not generally pathogenic to man. However, the World Health Organization includes them in the list of aesthetic nuisance and indicators of water treatment plant efficiency (Matsumoto et al. 2002). Despite that, certain species of nematodes are human intestinal parasites such as *Ascaris lumbricoides* but more frequently, they are vectors of human or animal pathogenic bacteria.

1.4.1 Survival of microorganisms inside nematodes

There are several reports on the ability of nematodes to ingest bacteria, to favour their persistence in the environment and therefore serve as potential vectors for pathogenic organisms. Interestingly, information about this specific problematic can be taken not only from drinking water related studies but also from research in the field of agriculture and food production. In fact, many scenarios in food and agriculture industry can lead to the need to study nematodes as pathogenic bacteria vectors. For instance, certain nematode species which are pathogenic to plants are controlled with bacteria that are pathogenic to nematodes, (Chen et al. 2000), whereas certain nematodes can be beneficial in agriculture as biological control agent, acting as vectors of bacteria that are pathogenic to other organisms that are harmful to plants (Tan and Grewal 2001).

More importantly in the context of this review, researchers became interested in nematodes as vectors of bacteria that are pathogenic to humans to evaluate the health risk associated to consuming raw fruits and vegetables. These researchers evaluated resistance to disinfectants in bacteria ingested by nematodes, therefore providing information that can be useful in a health risk assessment related to the presence of nematodes in drinking water systems, which information was therefore included in this section.

In the field of drinking water, Chang and his co-workers have studied long ago the health significance of nematodes with regard to their ability in protecting bacteria. They found that certain species of nematodes could ingest pathogenic bacteria such as *Salmonella* and *Shigella* as well as Coxsackie virus and echovirus, and up to 16% of the ingested organisms could survive for 24 hours at 25^{o}C inside their host (Chang et al.1960b). Conventional treatments were observed to be ineffective in terms of removal or inactivation of nematodes, despite their

sedimentation being facilitated when they lose their motility, which is possible following chlorination at 180 mg·min/L (Chang 1961), a treatment condition too severe to be applied at the head of a treatment plant. Nematodes were observed to survive chlorination as high as 360 mg·min/L (Chang 1961).

Nematodes of the *Rhabditidae* family can originate from wastewaters and can therefore transport and protect enteric bacteria and viruses, presenting therefore a potential health risk if found in drinking water systems (Chang et al. 1960b). In a study about wastewaters, nematodes isolated from trickling filters effluents were found to contain about 100 viable bacteria per nematode, while an average of 75 viable bacteria per nematode was counted in the effluent of a primary settler in a wastewater treatment plant (Chang and Kabler 1962). Approximately 5 to 10% of these bacteria were coliforms, and bacteria such as *E. coli*, *Pseudomonas* sp., *Streptococcus* sp. were identified, among others.

The nematode *Caenorhabditis elegans,* a member of the *Rhabditidae* family that feeds non selectively, has been used in several studies to investigate host-pathogens interactions. An exhaustive list of known pathogens of *C. elegans,* including various opportunistic and true human pathogens, is found in Sifri et al. (2005). *C. elegans*, used as a model host in agriculture studies, and the nematode *Diploscapter* sp., commonly found in agricultural soil and in compost, can vector various strains of *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* (Caldwell et al. 2003b), as those nematodes are attracted to the pathogenic bacteria and are able to ingest and transport them in their digestive tract (Gibbs et al. 2005). *C. elegans* has also been shown to transmit bacteriophages from one bacterial colony to another on a Petri dish (Dennehy et al. 2006). Furthermore, when it is pre-exposed to a bacteriophage population, *C. elegans* can better survive in the presence of *Salmonella enteritidis* and *Salmonella pullorum* (Santander and Robeson 2004), suggesting that the phages remain viable and active inside the nematode's gut after ingestion.

C. elegans was reported to ingest, transport, and excrete *Cryptosporidium parvum* oocysts (Huamanchay et al. 2004) and, in experimental conditions, 75 to 85 % of nematodes ingested up to 200 oocysts after two hours of incubation. The ingested oocysts remained intact, viable, and

infectious within the digestive tract of the nematode, with possible excystation and freeing of sporozoite into the gastro-intestinal system of the host. Nematodes that contained oocysts and that had been exposed to desiccation for a day were able to cause infection in mice, while oocysts or nematodes alone having undergone the same treatment did not infect the mice. Furthermore, *C. elegans* containing *C. parvum* oocysts had the ability to infect mice even after having been kept in water for 7 days. However, the nematodes were exposed to unreasonable levels of oocysts (2×10^6 oocysts for 100 to 200 nematodes) compared to the anticipated conditions in surface water or granular filter water, for example. Our work (unpublished) suggests that *C. elegans* does not spontaneously seek to feed on *C. parvum* oocysts, especially if other particles are available for its feeding, and oocyst ingestion seems to occur rather fortuitously. It would therefore seem highly unlikely to find an oocyst within a nematode in nature. In the perspective of performing a risk assessment, it seems more appropriate to focus on nematodes as vectors of pathogenic bacteria rather than oocysts, since the risk associated with infectious oocysts being carried inside nematodes is likely to be very low.

1.4.2 Protection of ingested microoorganisms by nematodes against water treatment

Cocultures of nematodes and bacteria *Salmonella typhi* and *S. wichita* were exposed to 10 mg/L of free chlorine for 15 minutes and viable bacteria were released after ingestion and natural defecation by nematodes (Smerda et al. 1970). Chlorine treatment killed all bacteria attached to the surface of nematodes, whereas, depending on the culture medium used after chlorine exposure, the recovery of *Salmonella* varied from 20 to 93.3%. Viable *S. wichita* were freed from nematodes in a tap water solution by defecation, which reflects drinking water conditions and therefore the potential health risk linked to its consumption. Bacterivorous nematodes can excrete from 30 to 60 % of bacteria ingested in viable form (Chantanao and Jensen 1969).

Nematodes of the genus *Rhabditis*, which are commonly found in drinking water distribution systems, were shown to provide protection to *E. coli* C600 against free chlorine at doses of 0.5 and 1.0 mg/L (Ding et al. 1995). *E. coli* freed from the nematodes by ultrasound and exposed to chlorine for 15 minutes were reduced by 6 logs, while bacteria that were protected by nematodes

were only reduced by 2.5 logs under the same conditions. It is possible that this 2.5-log reduction is due to bacteria being attached to the surface of nematodes, since following an hour of exposure to chlorine, the concentration of recovered bacteria was the same as after 15 minutes. It begs the question of whether or not the ingested *E. coli* had completely resisted the chlorine treatment, while *E. coli* attached to the nematode cuticle had been disinfected almost as easily as bacteria suspended in water. Furthermore, bacteria ingested by nematodes were shown to survive chlorine exposure (2% v/v or 1050 mg/L) and were subsequently freed by nematode fecal waste, while bacteria alone or on the surface of nematodes did not resist the same treatment (Adamo and Gealt 1996).

Nematodes were collected from raw and treated waters of a drinking water plant using surface water (Lupi et al. 1995). Most of them were in larval stage and measured, on average, 45 micrometers in length. Those nematodes were exposed to 10 mg/L of free chlorine (NaOCl) for 10 minutes, so as to kill the bacteria attached to the surface of the nematodes, and were then mechanically grinded. Heterotrophic bacteria and enterobacteria were recovered from nematodes collected from both raw and treated water, yet in significantly lower quantities in treated water than in raw water (average values are 251 HPC and 11 enterobacteria per nematode in raw water samples compared to 6.3 HPC and 2.1 enterobacteria per nematode in clean water). *C. elegans* was observed to disperse the bacteria *E. coli*, *Salmonella typhimurium*, *Listeria welshimeri and Bacillus cereus* by excreting viable cells after ingestion and exposure to 3 mg/L of sodium hypochloride for 5 to 6 minutes (Anderson et al. 2003). Some chemical disinfectants used in agriculture, including free chlorine (at concentrations of 0.02 to 0.50 mg/L and contact time of 5 minutes) can inactivate the bacteria *Salmonella* Poona present on the surface of the nematode *C. elegans*, but not those that had been ingested (Caldwell et al. 2003a).

Some species of bacterivorous nematodes can provide protection to potentially pathogenic bacteria in natural and filtered waters as well as in distributed water. Bacteria protected by nematodes seem to be present in treated water in a concentration that is too low to be considered a real risk to human health (Lupi et al. 1995). Even though infectious doses are generally higher for bacteria, it is not definite that this conclusion can be extended to protozoa such as *Giardia* and

Cryptosporidium, or to viruses, which have very low infectious doses. Consequently, we suggest that nematodes be considered as an increased risk factor in water systems. Finally, let's note that pathogens protection against UV disinfection within nematodes has not been researched in the literature to this date.

1.5 Discussion

The internalization of microorganisms by higher organisms can be considered in various contexts of more or less significance in regards to public health. Ecologists are studying this phenomenon in an attempt to understand aquatic trophic networks and variations in the composition of microbial communities in surface waters, without considering specifically pathogenic microorganisms and without particularly trying to establish a link between these observed natural phenomena and human health. On the contrary, in the agricultural industry, the internalization of microorganisms by higher organisms is clearly related to health issues, offering solutions in certain cases, but causing problems in other cases. Meanwhile, drinking water specialists too often dissociate microorganisms' inactivation kinetics by disinfection processes from the natural conditions under which they take place. This review aims at (1) evaluating the scientific observations of the potential health risk arising from the diverse associations between superior organisms and pathogenic microorganisms in a drinking water perspective and (2) identifying the missing data which impedes the establishment of cause and effect relationships that would permit to better appreciate the sanitary risk associated with this phenomenon.

When considering the study of this phenomenon in the specific context of drinking water, the focus, in a perspective of risk analysis, has to be on microorganisms that are pathogenic to humans. The health risk associated with these pathogens being protected by higher organisms in drinking water should be considered at three steps of drinking water production: (1) at the source, especially in the case of unfiltered surface waters, (2) at the effluent of water treatment plant filters, since practically all granular media filters, whether or not they are used in a biological mode, are colonized by invertebrates, and (3) in the water distribution system, as suggested by the well-known case of *Legionella pneumophila* which proliferates in distribution systems in the presence of amoebae.

The first clue of the significance of higher organisms in water systems can be found in studying their occurrence at those three stages of water production. At the source, for instance, concentrations between 2 and 3,000 amoebae per litre, and from 200 to 90,000 amoebae in river water were found during a three-year investigation in Germany, in untreated reservoir water and in rivers used as water supply sources, respectively (Hoffmann and Michel 2001). Densities of 200 to 300 rotifers per liter are common in natural freshwaters and can occasionally reach 1000 per litre (Wetzel 2001). As of filters, important densities of nematodes were measured in sand samples taken near the surface of a slow sand filter bed (approx. 570 nematodes in a 30 g sand sample), as well as other types of zooplankton organisms such as amoebae, rotifers and copepods (approx. 140 amoebae, similar quantity of rotifers and about 60 copepods in a 30 g sand sample) (Hijnen et al. 2007). Invertebrate concentrations, mainly nematodes or rotifers, in the order of several thousands individuals per litre have been reported at the effluent of granular filters (Schreiber et al. 1997, Castaldelli et al. 2005) and concentrations of up to 400 amoebae per litre were measured in filtered water from drinking water purification plants (Hoffmann and Michel 2001). As for distribution systems, protozoa are thought to be present in most systems in concentrations between $5x10^4$ et $7x10^5$/L (Sibille et al.1997) whereas cladocerans and copepods have been found in concentrations between 600 and 750 organisms/m^3 in samples taken at hydrants (van Lieverloo et al. 1998).

However, the sanitary significance of higher organisms in water is determined by the presence of internalized waterborne pathogens, whose resistance to primary or secondary disinfection may be enhanced. Invertebrates in distribution systems were found to be colonized by a large variety of bacteria, in numbers ranging from 1 to 10 CFU/copepod and from 10 to 100 CFU/nematode, both inside their digestive system and on their surface where the bacteria could be found individually or in colonies (Levy et al. 1986). Average colony counts reaching up to 4000 bacteria per invertebrate were observed in a drinking water system, approaching the infectious dose for certain of the bacterial species identified, possibly associated with a single superior organism (Wolmarans et al. 2005). Some of the invertebrate-associated bacteria from that system were identified to be frank or opportunistic human pathogens (including *Aeromonas hydrophila,*

Burkholderia cepacia, Klebsiella pneumonia, Pseudomonas aeruginosa, Enterococcus faecium, Streptococcus agalactiae, to name only a few). Moreover, total coliforms, atypical coliforms and heterotrophic aerobic bacteria (HPC) were shown to be released from nematodes when they transited through the high-pressure pumps of a drinking water distribution system (Locas et al. 2007), explaining the seasonal recurrence of total coliform bacteria at the volute of pumps (even though free chlorine residuals were as high as 1 mg Cl_2/L). In that specific case, the free chlorine residual maintained in the distribution system allowed for the rapid inactivation of the released bacteria, limiting the potential microbial risk. These studies investigating the association of bacteria with invertebrates in distribution pipes, even though most of them don't identify pathogens, are interesting in the way that they report viable bacteria that are really found to be associated with higher organisms in the distribution network, which is the most crucial location in terms of risk of transmission to humans. Of course the unpathogenic bacteria are insignificant in terms of health risk, and there is a need to better identify those invertebrate-associated bacteria in order to find out in what proportion do pathogens occur in association with invertebrates in real conditions.

Meanwhile, it is estimated that approximately 25% of *Acanthamoeba*, isolated as much from the environment as from humans, carry endosymbionts (Winiecka-Krusnell and Linder 2001). Amoebae infected by bacteria seem to be common in cooling towers (present in 22 /40 samples) whereas they seem more rare (or perhaps harder to detect) in natural aquatic environments (present in 3/40 samples), as shown in a study characterizing 40 samples of water, biofilms and sediments from cooling towers in various American states, as well as 40 samples from various lakes, rivers, creeks, and ponds (Berk et al. 2006). Amoebae were found to be infected mostly by bacteria other than *Legionella pneumophila*, most of which were not culturable outside of an amoeba.

Altogether, these studies lead us to consider the large group of superior organisms as vectors for human pathogenic microorganisms, a reality which we cannot permit ourselves to ignore in the drinking water production industry, considering that a single organism could potentially transmit an infectious dose through a drinking water distribution system. However, very few information

is currently available in scientific literature to quantify the risk associated with this issue. A quantitative microbial risk analysis model was developed for *Legionella erythra* in drinking water distribution systems. This species was used as a substitute for the human pathogen *L. pneumophila* (Storey et al. 2004a). It was shown that the presence of *Acanthamoebae castellanii* was an important risk factor for Legionnaires' disease, increasing the bacterium's resistance to free and combined chlorine, as well as to thermal treatment. An increased risk of approximately two orders of magnitude was calculated in the presence of amoebae, compared to the risk associated with planktonic bacteria exposed to the same thermal treatment conditions or free or combined chlorine disinfection conditions. In a similar analysis, the calculated risk was increased by one order of magnitude when considering bacteria associated to biofilm, suggesting that amoebae would be a more important risk factor for *Legionella* in water systems than biofilm attachment. In practice, it is difficult to quantify the risk of Legionnaires' disease caused by inhalation of the bacteria by users of the drinking water distribution system given, on one hand, the obvious lack of available data regarding the dose-response relationship associated with exposure to *Legionella*, and, on the other hand, the approximation necessary in evaluating volume of water particles inhaled by a user. In this study (Storey et al. 2004a), the maximal risk approach was used, which assumes that exposure to a single pathogenic microorganism results in an infection in the host, which can be justifiable when considering the worst case scenario, which would be that of an immunodeficient individual during a nosocomial contamination episode. All other values entered in the risk analysis model obviously call for estimation, for example, the normal adult respiration rate and the average duration of a shower, which is assumed to be the critical situation for maximum exposure to microorganisms carried in aerosol. Thus, the value of the calculated risk in this model is more relative than absolute. The exercise reported in this study highlights missing information necessary to more fairly evaluate the risk associated with the presence of *Legionella* in drinking water distribution systems, but it also allows to compare different strategies for the treatment and distribution of water in the context of reducing the risk of Legionnaires' disease while keeping in mind the ecological factors actually occurring in the distribution network. It is important to consider that the risk associated to amoebae harbouring *Legionella* in distribution systems must be considered in terms of inhalation, whereas amoebae could transmit human enteric pathogens such as *Campylobacter* through the usual route of exposure, *i.e.* by water consumption.

In surface waters, harsh environmental conditions seem to favour the association of some bacterial pathogens with higher organisms. In fact, endosymbiotic relationships between amoebae and bacteria or viruses can take place as a survival strategy used by endosymbionts. Similarly, it is thought that attachment of *Vibrio cholerae* to the surface of planktonic organisms in water can also be seen as a survival mechanism in hostile conditions, since the bacteria can find a rich nutrient source in the chitinous surface of some zooplankton organisms. This can be compared to the formation of biofilms, possibly on a biological surface, known to occur in V. cholerae for instance in reaction to grazing pressure by protozoa (Matz et al. 2005). Such microbiological behaviours, which enhance the survival time of human pathogens in the environment, are usually not the object of engineering concerns, therefore the contribution of microbiologists, biologists and ecologists is crucial. Water engineers should, for their part, focus on gathering quantitative information in a risk assessment perspective. One flaw found in literature in that regard is the lack of information about the resistance of internalized pathogens to various disinfection processes, including UV, which efficiency is known to be influenced by physical embedment of microorganisms in water. Specifically, we found that resistance to disinfection of pathogens located inside zooplankton organisms such as rotifers, copepods and cladocerans has not been assessed to date. Such studies can be performed in artificial conditions, using model hosts for instance, since it is difficult to isolate one species of zooplankton organisms from natural water samples. However, it is also important to assess the significance of higher organisms in water systems by performing field experiments. In fact, we are under the impression that laboratory experiments consisting in feeding higher organisms with pathogens, such as *Cryptosporidium* and *Giardia*, for instance, can be considered as misleading to some extent, or at least incomplete, since artificial conditions of highly improbable occurrence in water systems are necessary in order to observe ingestion. Such studies can however be revealing if they are paired with observations, in field experiments, of that same host-pathogen association. We therefore suggest that studies such as those performed by King et al. (1991) and Nowosad et al. (2007) on zooplankton harbouring microorganisms, and that of Berk et al. (2006) on infected amoebae, for instance, be put forth, to detect viable pathogens naturally occurring inside of higher organisms in water samples. In order to detect internalized human pathogens, it is necessary to sample highly contaminated waters. It is also important to include, in such studies, a

relevant assessment of the ability of the recovered pathogens to infect a human host. Additionally, we deem important to better investigate the occurrence of viable pathogens located inside invertebrates in water distribution systems, similarly to the work of Wolmarans et al. (2005), since the abundance of invertebrates in the pipe systems is well known, while their sanitary significance remains poorly characterized and addressed.

In practical terms, the time factor is a major challenge in assessing the microbial risk associated to higher organisms as vectors of human pathogens in water. The ingestion of pathogens by some invertebrates can be studied as a removal mechanism, for example in granular filters, if pathogens are digested following ingestion. In fact, many biological processes, used in wastewater treatment for instance, rely on higher organisms digesting waterborne pathogens. It is therefore natural to anticipate that the fate of most ingested pathogens is to be eliminated from water. However, studies reported in this review about nematodes vectoring human pathogens, for example, prove that on the contrary, some invertebrates can in some instances get associated (internally or externally) with human pathogens and carry them through water systems. It is thought that the time factor is important to consider in such cases, since pathogens may not be digested if water consumption by human occurs shortly after pathogen ingestion by zooplankton and nematodes. Moreover, most of the invertebrates that are released in the filters effluents are offsprings of the populations growing in the filters, and therefore and most probably not infected with pathogens from the source water. Altogether, this would suggest that the main risk associated to invertebrates in distribution system would result from the ingestion of pathogens in the distribution mains, a situation which is less probable than in the case of contaminated surface waters. Laboratory experiments have also allowed highlighting the importance of this time factor when bacteria and amoebae are in coculture. In fact, in some cases, a prolonged incubation could lead to the loss of viability in the intracellular bacteria, whereas in other cases, it could result in the destruction of the host cell. The case of bacteria that replicate inside protozoan hosts is probably the most important concern in terms of health risk management for drinking water, since the number of bacteria contained in a single organism can easily exceed the infectious dose for humans. It is also of concern to know that those bacterial pathogens can survive within the resisting form of their host, i.e. the cysts, considering that cysts can resist various extreme conditions and have a prolonged survival time in natural environments and engineered water

systems. We can however consider the more specific case of bacterial pathogens replicating inside protozoa separately from the more general case of pathogens ingestion by invertebrates, which most often imply a probability of digestion by the host with time. In fact, when taking this time factor into account, it appears that the sum of conditions that are needed to occur simultaneously at a specific location in order to observe a significant sanitary risk make this situation more of a coincidence of low probability, except for the case of bacterial replication inside protists. In fact, intracellular pathogens are more likely to persist throughout all steps of the transmission route from the treatment plant to humans, and that, in a sufficient number to possibly create an infection in human after ingestion of inhalation.

It is important to consider the limits of the experimental methods that are used when assessing the survival of microorganisms inside higher organisms and their resistance to disinfection. One of the greatest challenges concerns the necessity to prove whether or not pathogens that survive within a higher organism are able to create an infection in human cells. Microscopic observation is often ambiguous on that aspect, whereas standard culture methods can also be misleading, since some bacteria have a viable but not culturable state in which they remain viable. Furthermore, many bacteria found to infect amoebae are not culturable outside of amoebal hosts (Berk et al. 2006). However, it was shown that many bacterial pathogens resist digestion within protozoa by using similar mechanisms as the ones used to infect human macrophages. Therefore, it is interesting to explore whether the use of amoebae could be relevant when assessing the viability of such human pathogens, since replication in amoebae could indicate the ability of bacteria to cause an infection in a human host.

The few cases of pathogenic microorganisms being detected within a host might be clues to a phenomenon that is more widespread than acknowledged by the drinking water treatment industry. The detection of viable *Cryptosporidium* oocysts within rotifers in natural lake waters (Nowosad et al. 2007) could lead to question the validity of the pathogenic microorganism concentrations measured during microbiological characterization of surface waters, which does not usually take into consideration microorganisms that are potentially viable within higher organisms. Some may argue that the occurrence of pathogens protected inside of higher organisms is a rare event in treated waters from a conventional treatment plant. But the

objectives in treating drinking water in North America require, in practice, the production of water that contains less than one parasite per 100 000 L (USEPA 2006). In this context, the presence of viable parasites inside higher organisms, even though it is, according to all evidence, a rare phenomenon, could potentially be a non negligible risk, keeping in mind the protection that this higher organism brings to the pathogenic organisms it harbours. To date, the required information to properly evaluate this risk remains incomplete or missing.

The study of *Legionella* and its mechanisms for resisting digestion by an amoeba, for example, opens the door to the analysis of a more general health risk that might possibly result from the high disinfection resistance of pathogenic bacteria protected by a protozoa or one of its vesicles. Even though predation by higher organisms can be more intense at certain steps in a water treatment system, for example in granular filters where benthic invertebrates such as nematodes proliferate, an evaluation of the importance of the phenomenon of pathogen internalization by higher organisms in raw water is completely absent in the scientific literature. Despite the quantification obviously presenting major methodological challenges, such work would provide a valuable knowledge to the water industry. If the concern for public health of drinking water scientists could be combined with the interests of research in freshwater ecology, a significant scientific contribution could arise from such collaboration. The study of the resistance of internalized pathogens to traditional disinfectants as well as to advanced treatments such as UV disinfection is imperative to the drinking water industry and to the evaluation of the microbial risk. The emergence of UV disinfection could prove to be an interesting tool if UV rays can successfully inactivate microorganisms harboured by higher organisms, a demonstration that has not yet been done to this day.

While we are starting to understand the resistance mechanisms of microorganisms exposed to disinfection, such as the forming of biofilms, aggregation, and attachment to particles or to surfaces, biological or not, the study of microorganism survival within higher organisms proves to be necessary so that we may no longer ignore the field conditions that characterize natural environments and that are too often excluded from laboratory disinfection assays, which nevertheless determine to this day the disinfection standards for the drinking water industry.

Acknowledgements: The authors would like to acknowledge the NSERC Industrial Chair on Drinking Water and its industrial partners, namely the City of Montreal, John Meunier Inc and the City of Laval. Special thanks to Normand Labbe for its support, to Ana Esquivel and Tyler Ball for their help with translation and to Annie Locas and the anonymous reviewers for their helpful comments.

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CHAPITRE 2 OBJECTIFS DE RECHERCHE ET DÉMARCHE EXPÉRIMENTALE

Afin d'explorer la question du risque sanitaire associé aux microorganismes pathogènes internalisés par des organismes supérieurs dans l'eau potable, une revue critique de la littérature a d'abord été réalisée afin de dresser le portrait le plus complet et le plus à jour possible des connaissances du monde scientifique sur les éléments participants à ce risque. Cette revue critique présentée au chapitre précédent avait également pour fonction d'identifier les principales inconnues dans l'évaluation de ce risque microbiologique. Dans le but de préciser le problème à l'étude dans le cadre de cette thèse, les aspects les mieux documentés dans la littérature ont été écartés ou du moins abordés seulement en périphérie des expériences élaborées. En effet, suite à une synthèse de la littérature, les tendances suivantes ont pu être soulignées : (i) Les interactions entre les protistes, particulièrement les amibes, et des bactéries pathogènes humaines font l'objet d'études spécifiques dans différents contextes de recherche, incluant des études poussées en microbiologie sur les mécanismes intracellulaires des bactéries internalisées et mettant en évidence l'importance des ces phénomènes au niveau du risque sanitaire. Ceci constitue une incitation à traiter les protistes, organismes unicellulaires, comme un groupe distinct des autres hôtes, soit les organismes multicellulaires du zooplancton (rotifères, copépodes, cladocères, considérant ici également les nématodes), dans la problématique de l'internalisation des microorganismes dans l'eau potable. (ii) L'occurrence des organismes supérieurs en eau potable est principalement caractérisée à l'effluent des filtres granulaires et en réseau de distribution. (iii) Les organismes du zooplancton, entre autres les rotifères et les cladocères, peuvent ingérer, en conditions de laboratoire, des microorganismes pathogènes résistants et d'importance cruciale en eau potable tels que les protozoaires *Cryptosporidium* et *Giardia*. (iv) Des observations du rôle des nématodes comme prédateurs et véhicules de bactéries pathogènes humaines sont abondamment rapportées dans la recherche en eau potable et en industrie agro-alimentaire, incluant la capacité des nématodes de protéger les bactéries ingérées contre la désinfection chimique, permettant de supposer entre autre une protection pratiquement complète contre la chloration dans des conditions typiques d'eau potable.

À partir de ces tendances observées dans la littérature, les objectifs de la présente thèse ont été précisés, tels que décrits à la section suivante.

2.1 Objectifs de recherche

L'objectif général de ce projet de recherche consiste à évaluer l'impact de l'internalisation des microorganismes pathogènes par les organismes supérieurs sur le risque microbiologique lié à la consommation d'eau potable.

L'évaluation de l'impact de l'internalisation des microorganismes pathogènes par les organismes supérieurs nécessite de considérer plusieurs aspects du phénomène qui sont encore peu documentés dans la littérature scientifique, soit principalement: (i) la probabilité d'occurrence d'un microorganisme pathogène à l'intérieur d'un organisme supérieur dans l'eau potable; (ii) l'impact des procédés de désinfection sur la survie d'un microorganisme internalisé par un organisme supérieur.

La filtration granulaire a été ciblée comme un milieu favorable à l'internalisation des microorganismes, entre autre parce que les organismes supérieurs y sont abondants et que leurs activités de prédation y sont intensifiées. La désinfection UV a été identifiée comme un procédé de traitement de l'eau potable présentant un potentiel d'inactivation des microorganismes internalisés par les organismes supérieurs, en contraste avec la plupart des désinfectants chimiques rapportés comme relativement inefficaces face à cette problématique. Enfin, l'analyse quantitative du risque microbien (QMRA) constitue un outil de plus en plus standardisé en eau potable pour évaluer dans une approche probabiliste divers scénarios associés à un risque d'infection chez les consommateurs.

Cette thèse se donne pour objectifs spécifiques de :

(1) caractériser l'occurrence du phénomène d'internalisation de microorganismes pathogènes par les organismes supérieurs dans la filtration granulaire et le transport des organismes internalisés dans l'eau filtrée;

(2) caractériser l'effet de protection dont bénéficient les microorganismes internalisés par des organismes supérieurs sur la performance de la désinfection UV;

(3) comparer l'effet de protection dû à l'internalisation des microorganismes parallèlement à celui de l'agrégation et de l'attachement aux particules sur la performance de deux

procédés voisins, soit la désinfection UVC et la désinfection solaire (UVA), appliqués respectivement dans les pays industrialisés et en développement;

(4) développer, par la caractérisation d'une chaîne d'événements décrivant l'internalisation et le transport de microorganismes par les organismes supérieurs, un modèle d'analyse de risque permettant de quantifier le risque sanitaire associé aux microorganismes pathogènes internalisés dans l'eau potable.

La réalisation de ce projet de thèse repose sur les hypothèses de recherche suivantes :

(1) Des microorganismes pathogènes résistants tels que les (oo)cystes de protozoaires peuvent être internalisés par des organismes du zooplancton dans des filtres granulaires et peuvent être partiellement transportés comme organismes internalisés dans l'effluent filtré.

(2) Les microorganismes internalisés par des organismes supérieurs sont partiellement protégés contre les radiations UV (254 nm) grâce à leur hôte et sont donc inactivés dans une moindre mesure par rapport à des microorganismes libres face à une même dose UV.

(3) Les mécanismes de protection des microorganismes tels que l'agrégation, l'attachement aux particules et l'internalisation par les organismes supérieurs ont des impacts comparables face à la désinfection UV et la désinfection solaire (UVA) de l'eau potable.

(4) L'internalisation de microorganismes pathogènes résistants tels que les (oo)cystes de protozoaires est un phénomène rare en eau potable, mais qui peut potentiellement représenter un risque sanitaire considérant les faibles concentrations prescrites par la réglementation courante, et posant qu'un (oo)cyste internalisé et rejeté à l'effluent d'un filtre granulaire présente une forte probabilité de survivre à travers les barrières de désinfection subséquentes tant qu'il demeure à l'intérieur de son hôte.

2.2 Méthodologie

Le plan expérimental élaboré pour cette thèse aborde ainsi deux axes de recherche découlant de l'identification des principales lacunes dans la littérature au sujet des microorganismes pathogènes internalisés par des organismes supérieurs dans l'eau potable, soit (i) la caractérisation de l'occurrence et du transport des protozoaires *Cryptosporidium* et *Giardia* internalisés par des organismes du zooplancton dans des conditions environnementales en eau potable; (ii) l'évaluation de la protection de microorganismes internalisés par des organismes du zooplancton contre la désinfection UV, considérant son utilisation typique en aval d'une filtration granulaire, où la prédation par les organismes supérieurs (et donc l'internalisation) est favorisée.

Ces deux axes de recherche ont été développés au niveau expérimental de la façon suivante : (i) l'occurrence des protozoaires *Cryptosporidium* et *Giardia* internalisés par des organismes du zooplancton a été étudiée dans des conditions de filtration granulaire à l'échelle pilote, avec ensemencement de hautes concentrations d'(oo)cystes de *Cryptosporidium* et *Giardia*, et (ii) la protection de microorganismes internalisés par des organismes du zooplancton contre la désinfection UV a été évaluée à l'échelle du laboratoire à l'aide de bactéries indicatrices telles que *E. coli* et les spores de *Bacillus subtilis* internalisés par des nématodes, soit un système biologique préalablement utilisé par d'autres auteurs (Ding et al. 1995, Adamo et Gealt 1996, Anderson et al. 2003, Kenney et al. 2005, Laaberki et Dworkin 2008) pour étudier la protection de bactéries internalisées face à la désinfection chimique. À ces travaux sur la désinfection UV, se sont ajoutés des travaux parallèles visant à comparer l'impact mesuré dû à l'internalisation des microorganismes face à la désinfection UV (254 nm) avec l'impact d'une telle protection dans la désinfection UVA (solaire), un procédé de désinfection voisin de la désinfection UV, basé sur l'action des radiations UVA naturelles du spectre solaire et appliqué dans les communautés en développement. Cette étude parallèle s'est étendue à l'évaluation plus large des mécanismes de protection des microorganismes face à la désinfection UVA, en comparaison avec la désinfection UV (254 nm) : l'impact des mécanismes d'agrégation naturelle des microorganismes et de l'attachement aux particules a été inclus dans ces travaux.

Enfin, l'analyse quantitative du risque microbiologique (QMRA) a été utilisée en étape de synthèse de cette recherche afin de rassembler, d'ordonner et de quantifier le plus possible de morceaux du casse-tête pour répondre à la question suivante : « Quel est le risque sanitaire

associé aux microorganismes pathogènes internalisés par des organismes supérieurs dans l'eau potable ? » Le risque sanitaire a été estimé quantitativement par des simulations Monte-Carlo basées sur le modèle exponentiel d'infectivité, sachant que, selon les pratiques réglementaires actuelles de la USEPA, la probabilité d'infection annuelle $P_{inf\ (annuelle)}$ ne doit pas excéder 10^{-4}, soit une infection par 10 000 personnes annuellement.

La méthodologie développée pour chacune des phases expérimentales de cette thèse est détaillée dans les sections suivantes.

2.2.1 Occurrence et transport de microorganismes pathogènes internalisés par des organismes supérieurs dans la filtration granulaire

Les (oo)cystes de *Cryptosporidium* et *Giardia* ont été choisis comme microorganismes pathogènes résistants permettant d'étudier l'internalisation et le transport des microorganismes dans la filtration granulaire. En effet, l'ingestion d'(oo)cystes par les organismes du zooplancton (rotifères, daphnies, nématodes, protozoaires ciliés) a été observée en laboratoire (Fayer et al. 2000, Trout et al. 2002, Stott et al. 2001, Stott et al. 2003, Huamanchay 2004, Connelly et al. 2007) et des oocystes de *Cryptosporidium* internalisés dans des rotifères ont été détectés dans des conditions complètement naturelles d'eaux de lacs (Nowosad et al. 2007). Une étude a été réalisée à l'échelle pilote afin de permettre l'ensemencement des filtres à des concentrations élevées d'(oo)cystes de *Cryptosporidium* et *Giardia*. Des colonnes de filtration au charbon actif granulaire (CAG) naturellement colonisées de zooplancton ont été utilisées afin de favoriser l'observation de prédation dans le lit filtrant. Ces travaux comprennent principalement 3 parties expérimentales, soit (i) le développement d'un protocole permettant une rupture complète d'organismes du zooplancton par sonication dans le but de libérer et d'énumérer les (oo)cystes internalisés; (ii) le prélèvement d'échantillons de matériau granulaire (CAG) à différentes profondeurs du lit filtrant suite à l'ensemencement des filtres afin d'énumérer les (oo)cystes retenus dans le lit filtrant et attachés aux grains de charbon actif ainsi que les (oo)cystes rejetés à l'effluent filtré; (iii) l'isolement d'organismes du zooplancton dans les échantillons de CAG prélevés du lit filtrant et à l'effluent pour l'énumération du zooplancton et l'extraction et énumération des (oo)cystes internalisés par l'application du protocole de sonication précédemment optimisé.

2.2.1.1 Ensemencement des filtres CAG

Le montage de filtration CAG à l'échelle pilote est constitué de deux colonnes identiques en parallèle (15 cm de diamètre, 1 m de profondeur) opérées (sans rétrolavage) à une charge superficielle de 5 m/h. Le temps de contact dans les colonnes est de 12 minutes. L'ensemencement d'(oo)cystes de *Cryptosporidium* et *Giardia* est d'une durée de 2 heures à des concentrations de $1.6^{E}6$/L et $4.8^{E}4$/L à l'affluent, respectivement. Les (oo)cystes ont été préalablement inactivés par irradiation UV et ne sont pas marqués par fluorescence. Le charbon utilisé pour remplir les colonnes du montage pilote provenait de filtres à échelle réelle après 40000 volumes de lit filtrant d'opération des filtres. Le montage pilote est opéré à l'effluent d'un traitement conventionnel (coagulation et filtration rapide sur sable) dans une usine de traitement d'eau potable à échelle réelle alimentée par l'eau de la rivière Meuse (Pays-Bas) après rétention dans un réservoir.

2.2.1.2 Développement du protocole de rupture des organismes du zooplancton et de récupération des (oo)cystes internalisés

Des organismes du zooplancton ont été isolés du *Schmudzdecke* de filtres à sable lents et à l'effluent des colonnes de filtration CAG avant l'ensemencement des filtres afin de préparer des échantillons des 3 groupes d'organismes suivant déterminés comme pouvant présenter une résistance semblable à la sonication : (1) les nématodes, (2) les rotifères sans carapace rigide et (3) les rotifères avec une carapace rigide. Plusieurs filtrations successives sur un filet à plancton de 30 μm et plusieurs rinçages à l'eau non chlorée ont permis l'isolation du zooplancton, et la division par groupe a été réalisée en prélevant un à un les organismes de chaque groupe à l'aide d'une micropipette au binoculaire. Plusieurs échantillons de 5 ml d'eau stérile contenant chacun 20 organismes d'un même groupe ont été préparés. Le protocole de sonication a été optimisé en appliquant différentes durées de sonication à chaque échantillon de zooplancton et en mesurant le pourcentage d'organismes détruits par chaque traitement. Une sonde Branson Sonifier S-250D a été utilisée à des amplitudes de 45% et 65%.

2.2.1.3 Analyse des (oo)cystes libres retenus dans le lit filtrant

Une et trois semaines après l'ensemencement des filtres, des échantillons de CAG ont été prélevés à travers le lit filtrant des deux colonnes de filtration selon la distribution des

profondeurs suivante : couche supérieure, 0-5 cm, 5-10 cm, 25-30 cm, 50-55 cm, 85-90 cm. Des échantillons composites ont été préparés en combinant les échantillons des 4 couches supérieures (0-30 cm) et ees 2 couches inférieurs (50-90 cm). Les échantillons prélevés après 3 semaines ont été traités spécialement pour favoriser le détachement des (oo)cystes du matériau granulaire, en appliquant successivement les 4 étapes suivantes : 2 minutes d'agitation manuelle, 2 minutes de mélange au vortex, 2 minutes de sonication à faible énergie dans un bain à ultrasons, puis, 2 minutes de sonication à énergie élevée à l'aide d'une sonde (45% d'amplitude). Les (oo)cystes récupérés ont été isolés par IMS et énumérés par microscopie en épifluorescence (Leica, DM RXA) à un grossissement de 250X.

2.2.1.4 Analyse des (oo)cystes internalisés dans les échantillons de zooplancton prélevés du lit filtrant et à l'effluent

Les échantillons composites de matériau granulaire préparés tel que décrit plus haut ont servi également à l'isolement d'organismes du zooplancton pour l'analyse d'(oo)cystes internalisés dans les deux parties du lit filtrant (0-30 cm et 50-90 cm). Le zooplancton a été isolé du matériau granulaire et concentré grâce à un protocole basé sur la filtration sur un filet à plancton de 30 μm. Le zooplancton à l'effluent des colonnes de filtration a également été isolé sur un filet de 30 μm. Le traitement optimal déterminé plus tôt dans l'étude pour la rupture des organismes du zooplancton a été appliqué aux échantillons concentrés de zooplancton du lit filtrant et de l'effluent afin d'extraire les (oo)cystes internalisés. Les échantillons ont été divisés afin de comparer l'énumération d'(oo)cystes suite à la méthode IMS pour des échantillons de zooplancton non traités et des échantillons traités par sonication pour la rupture du zooplancton. Cette comparaison a permis d'évaluer la portion d'(oo)cystes internalisés correspondant à la hausse des comptes d'(oo)cystes par rapport aux échantillons non traités.

Les organismes du zooplancton ont été énumérés et identifiés par microscopie inversée (Leica, Leitz Labovert FS) à un grossissement de 100X pour les échantillons du lit filtrant et de l'effluent.

2.2.2 Protection face à la désinfection UV des microorganismes internalisés par des organismes supérieurs

L'impact de l'internalisation des microorganismes sur l'efficacité de la désinfection UV a été évalué en utilisant le nématode *C. elegans*, un modèle biologique répandu pour l'étude d'interactions hôtes-pathogènes, comme prédateur pour les bactéries *E. coli* et les spores de *B. subtilis*. Un protocole a été développé afin de (i) permettre la prédation des deux microorganismes ciblés (*E. coli* et spores de *B. subtilis*) par les nématodes, (ii) exposer les co-suspensions à l'irradiation UV (254 nm) et (iii) extraire les bactéries internalisées grâce à un protocole de sonication permettant la rupture des nématodes et l'énumération des bactéries par des méthodes de culture standards.

2.2.2.1 Préparation des co-suspensions de nématodes et proies

La souche sauvage N2 du nématode *C. elegans* a été utilisée dans cette étude. Les nématodes ont été cultivés sur des géloses d'agar NGM de 5 mm de diamètre. Une souche de *E. coli*-OP50 exprimant la protéine verte fluorescente (GFP) a été utilisée, ce qui a permis la visualisation des bactéries *E. coli* internalisées à l'intérieur du tube digestif des nématodes par microscopie en épifluorescence à des fins de contrôle qualitatif à différentes étapes du protocole. *E. coli* OP50-GFP a été cultivé à 37^{o}C pendant 24 h dans une solution L-Broth; 0.1 ml de cette culture a été déposé sur la surface des géloses de NGM avant une incubation à 37^{o}C pour 24 h permettant d'établir une croissance confluente des bactéries avant de transférer les nématodes sur les géloses. Les cultures de nématodes ont été synchronisées de manière à obtenir une population d'âge homogène pour les essais d'inactivation UV. Le protocole de synchronisation est basé sur l'exposition des nématodes à une solution contenant 5 ml de NaOH 1N et 2 ml de solution commerciale de chlore (hypochlorite de sodium ~5.25%) pendant 10 minutes, ce qui permet de tuer toutes les formes du cycle de vie des nématodes à l'exception des œufs. Suite à plusieurs rinçages à l'eau Milli-Q stérile et par centrifugation, les œufs sont déposés sur la surface des géloses NGM couvertes d'une couche de *E. coli* OP50-GFP. Les géloses ont été incubées à température pièce pendant 3 jours pour obtenir des nématodes adultes. Les nématodes récupérés sur la surface de 6 géloses après 3 jours d'incubation ont été utilisés pour chacun des essais d'inactivation UV.

Pour les essais d'inactivation des spores de *B. subtilis*, les nématodes adultes récupérés au 3^{e} jour d'incubation ont été rincés à plusieurs reprises par centrifugation avec une solution de tampon phosphate afin d'éliminer la plupart des bactéries *E. coli* externes (non ingérées par les nématodes) et favoriser l'ingestion subséquente de spores. Pour chacune des géloses utilisées, les nématodes rincés ont été resuspendus dans 5 ml de tampon phosphate stérile et 0.1 ml d'une solution de spores de *B. subtilis* (ATCC 6633, ~10^{8} UFC/ml) a été ajouté à la suspension. Chaque suspension a été transférée dans un Pétri stérile de 50 mm et incubé à température pièce pendant 3 heures pour permettre la prédation des spores par les nématodes.

À la fin des périodes de prédations pour *E. coli* et *B. subtilis*, les co-suspensions ont été rincées au tampon phosphate à plusieurs reprises par centrifugation et décantation afin de réduire la demande en chlore des échantillons, en prévision de l'étape subséquente du protocole.

2.2.2.2 Essais d'inactivation UV

Le protocole décrit ci-après a été entièrement répété trois fois, sur trois semaines successives, pour chacune des deux cibles *E. coli* et *B. subtilis*. Chaque réplicat a été réalisé à partir de nouvelles cultures de nématodes synchronisées. Les co-suspensions pour chaque essais ont été combinée en une seule suspension dans une solution de tampon phosphate d'un volume final de 50 ml. Après le rinçage pour la réduction de la demande en chlore, la suspension a été homogénéisée et la concentration initiale de bactéries et de nématodes a été mesurée. Ensuite, une étape de chloration a été appliquée aux suspensions afin d'éliminer les bactéries non internalisées par les nématodes: une solution d'hypochlorite de sodium a été appliquée aux co-suspensions nématodes-*E. coli* à 10 mg Cl_2/L pendant 5 minutes et à 15 mg Cl_2/L pendant 20 minutes pour les co-suspensions nématodes-*B.subtilis*. Les traitements de chloration ont été neutralisés par l'ajout de thiosulfate de sodium dans les deux cas. Après cette étape, les concentrations de *E. coli* et de spores *B. subtilis* ont été mesurées dans la solution avant de la diviser en deux parties égales : une partie serait traitée par sonication avant l'exposition à l'irradiation UV, ce qui permet de mesurer l'inactivation des bactéries extraites des nématodes, en présence de débris de nématodes, et l'autre partie serait traitée par sonication après l'exposition aux UV, permettant de mesurer l'inactivation atteinte pour des bactéries localisées à l'intérieur des nématodes pendant l'irradiation aux UV. Le traitement de sonication a été appliqué dans des tubes de verres sur glace à l'aide d'une sonde à ultrasons (Cole Parmer, CP

70T) opérée à une puissance de 15 W pendant 60 secondes. L'inactivation de solutions pures de *E. coli* et de spores de *B. subtilis* (planctoniques, en absence de nématodes) a également été mesurée pour des fins de référence.

Les suspensions ont été exposées dans des Pétri ouverts (9 cm de diamètre) à des fluences UV de 5 et 40 mJ/cm^2 en utilisant un montage de faisceaux collimatés équipé d'une lampe UV à basse pression (254 nm) (Trojan UV Technologies, London, ON, Canada). Le taux de fluence de la lampe a été mesuré grâce à un radiomètre (International Light Model IL1400A). La durée d'irradiation requise pour une fluence donnée a été calculée pour un taux de fluence corrigé selon la méthode standard proposée par Bolton et Linden (2003). L'absorbance UV (365 nm) a été mesurée avec un spectrophotomètre (Cary 100 Scan UV-Visible, Varian, Victoria, Australie) équipé d'une sphère intégrante (Labsphere, North Sutton, NH).

2.2.2.3 Énumération des bactéries et des nématodes

Les nématodes ont été énumérés en filtrant 1 ml de chaque suspension sur une membrane de 0.45 μm (Millipore, 47 mm), laquelle a été observée au microscope à un grossissement de 200X. La surface entière du filtre a été scrutée pour le compte de nématodes. Les bactéries *E. coli* ont été dénombrées sur des géloses d'agar m-Endo en duplicata après avoir filtré 1 ml des dilutions appropriées sur des filtres de 0.45 μm (Millipore, 47 mm). Les Pétri ont été incubés à 35^{o}C pendant 24h. Les spores de *B. subtilis* spores ont été dénombrés en filtrant les dilutions appropriées en duplicata et en transférant les filtres sur des tampons imbibés de TSB dans des Pétri. Une pasteurisation des Pétri à 75^{o}C pendant 15 minutes a été effectuée avant l'incubation à 35^{o}C pendant 24 h.

2.2.3 Comparaison des effets de protection dus à l'internalisation, l'agrégation et l'attachement des microorganismes dans la désinfection UV et solaire (UVA) de l'eau potable

Cette étude présente une évaluation comparative des mécanismes de protection microbiens dans la désinfection UV (UVC 254 nm) et solaire (UVA). Deux séries d'essais ont été entreprises afin d'évaluer l'impact de deux types de mécanismes de protection face à la désinfection solaire, soit (i) l'agrégation des microorganismes et l'attachement aux particules et (ii) l'internalisation des microorganismes par les organismes supérieurs. Ces travaux sont basés sur l'application de

protocoles de désinfection préalablement développés au sein de notre groupe de recherche pour évaluer l'impact de la turbidité (Caron et al. 2007) et de l'internalisation (tel que décrit sommairement à la section précédente) face à l'inactivation UVC. Des essais identiques ont été réalisés sous une irradiation UVA simulée en laboratoire afin de tester l'hypothèse que les effets protecteurs des deux types de mécanismes étudiés étaient similaires en désinfection UV et solaire.

Dans la première série d'essais sur la turbidité, des échantillons d'eau de rivière ont été traités de manière plus ou moins extensive afin de distinguer l'impact de la dispersion des agrégats et de l'enlèvement de particules sur l'efficacité de la désinfection UVA. Les coliformes totaux indigènes ont été sélectionnés comme microorganismes cibles pour ces essais puisqu'ils sont présents en concentrations suffisamment élevées dans les eaux de surface naturelles pour permettre la conduite d'essais de désinfection sans nécessiter l'ensemencement des échantillons d'eau. Cette approche est privilégiée afin de pourvoir des conditions plus représentatives des microorganismes tels qu'ils se retrouvent dans leur état naturel d'agrégation et d'association aux particules.

Dans les cas de l'internalisation microbienne, *E. coli* a été utilisé comme cible pour les essais de désinfection. Le nématode *C. elegans* a été utilisé comme un hôte modèle pour les bactéries internalisées. Les co-cultures ont été préparées en nourrissant les nématodes avec des *E. coli* sur des géloses d'agar. Les co-suspensions ont été préparées selon diverses étapes de prétraitement avant d'être exposées à l'irradiation UVA. Un protocole de sonication a été utilisé pour rompre les nématodes afin d'en extraire et récupérer les bactéries *E. coli* internalisées avant ou après l'exposition aux radiations UVA.

2.2.3.1 Irradiation UVA et mesure du taux de fluence

Les essais d'inactivation ont été réalisés à l'aide d'une lampe UVA (15 W) avec un pic d'émission à 365 nm (UVP, Upland, CA). Le taux de fluence a été mesuré avec un radiomètre (IL1400A, International Light, Newbury, MA). La durée d'irradiation requise pour une fluence donnée a été calculée pour un taux de fluence corrigé selon la méthode standard proposée par Bolton et Linden (2003). L'absorbance UV (365 nm) a été mesurée avec un spectrophotomètre (Cary 100 Scan UV-Visible, Varian, Victoria, Australie) équipé d'une sphère intégrante (Labsphere, North Sutton, NH). Pour permettre l'irradiation de plus grands volumes d'eau (50

ml), des Pétri de 9 cm de diamètre ont été utilisés. Avec cette configuration, la profondeur d'eau dans les échantillons était de 1 cm. Les suspensions ont été exposées aux UVA dans des Pétri ouverts et ont été doucement agitées par un barreau magnétique durant toute la durée de l'exposition.

2.2.3.2 Essais sur l'impact de l'agrégation et de l'attachement

Les échantillons d'eau brute ont été prélevés à la Rivière des Mille-Îles à 4 reprises au cours de l'été 2008. Chaque échantillon a été caractérisé pour les paramètres physico-chimiques suivants : turbidité, pH, alcalinité, dureté, comptes de particules, COT, absorbance UV à 254 nm (standard) et 365 nm (pic d'émission de la lampe UVA).

Le protocole utilisé pour évaluer l'impact de l'agréation des microorganismes et de l'attachement aux particules a été élaboré par Caron et al. (2007). Ce protocole permet une comparaison entre trois conditions: (i) les microorganismes non dispersés: les échantillons d'eau brute sont exposés à l'irradiation UVA sans aucun prétraitement, après quoi les échantillons sont dispersés dans un mélangeur (tel que décrit plus bas) afin de disperser les agrégats naturels de coliformes avant l'énumération; (ii) les microorganismes dispersés : les échantillons sont dispersés préalablement à l'irradiation UVA afin d'évaluer le rôle de l'agrégation des coliformes dans l'inactivation UVA; (iii) les microorganismes filtrés : les échantillons sont filtrés sur une membrane de 8 μm, après quoi les échantillons filtrés sont exposés à l'irradiation UVA et ensuite agités dans un mélangeur afin de disperser les coliformes avant l'énumération.

La procédure de dispersion est effectuée par un mélange vigoureux (Blender 7012S, Waring, Torrington, CT) des échantillons d'eau pendant 4 minutes à 8000 rpm avec 100 mg/L de Zwittergent 3-12 (Sigma Chemical Co., St-Louis, MO). Des intervalles de 2 minutes de repos sont alloués entre chaque minute de mélange afin de minimiser toute augmentation de la température de l'eau et la présence de mousse. Bien que ce protocole ait été développé originalement en ciblant les spores de bactéries sporulantes aérobies, une cible différente a été sélectionnée pour les essais d'inactivation UVA puisque les spores indigènes ne permettaient pas de mesurer des niveaux d'inactivation suffisants par les UVA pour permettre une comparaison des cinétiques d'inactivation pour les besoins de cette étude. En effet, les spores sont très résistants à la désinfection UVA (Boyle et al. 2008, Gill et McLoughlin 2007). Les suspensions ont été exposées aux radiations UVA pour différentes durées d'exposition, permettant d'atteindre

des doses UVA entre 10 et 35 J/cm^2. De telles fluences représentent une exposition à une forte radiation solaire pour des durées d'environ < 1 à 3 heures.

2.2.3.3 Essais sur l'impact de l'internalisation

Les essais sur l'internalisation des bactéries *E. coli* par les nématodes *C. elegans* ont été réalisés de manière identiques aux essais décrits à la section 2.2.2 de cette thèse et présentés en détails au chapitre 4: les nématodes ont été cultivés et nourris de la bactérie cible *E. coli* OP50 – GFP, les co-suspensions ont été exposées à l'irradiation UVA et une procédure de sonication a été utilisée afin de rompre les nématodes et en extraire les bactéries internalisées pour l'énumération par culture (dénombrement sur géloses). Le traitement de sonication a été effectué dans des tubes de verres sur glace à l'aide d'une sonde à ultrasons (Cole Parmer, CP 70T) opérée à 15 W pendant 60 secondes. La sonication a été effectuée soit avant, soit après l'irradiation UVA afin de comparer l'inactivation obtenue dans les deux cas. Les suspensions ont été exposées à des fluences de 0.70 et 5.60 J/cm^2. L'inactivation de solutions pures de *E. coli* (planctonique) a également été mesurée pour les mêmes fluences UVA pour des fins de référence. Les nématodes ont été comptés au microscope (200X) sur un filtre de 0.45 μm (Millipore, 47 mm). Les bactéries *E. coli* et les coliformes totaux ont été énumérés par filtration sur des membranes de 0.45 μm (Millipore, 47 mm) en duplicata incubées à 35^{o}C for 24h sur des géloses d'agar m-Endo.

2.2.4 Analyse quantitative du risque microbien associé aux microorganismes pathogènes internalisés dans l'eau potable

Cette étape de la thèse consiste en un exercice de synthèse sur la question du risque microbien associé aux microorganismes pathogènes internalisés dans l'eau potable. Ces travaux utilisent l'outil du QMRA (analyse quantitative du risque microbien) afin d'évaluer quantitativement la probabilité annuelle d'infection chez les consommateurs due aux microorganismes pathogènes internalisés par des organismes du zooplancton dans l'eau potable. Dans cette étude, la prédation des (oo)cystes de protozoaires par les rotifères dans la filtration granulaire a été ciblée comme problématique à l'origine du risque d'internalisation des microorganismes dans l'eau potable. À l'aide du logiciel Crystal Ball® (Decisioneering, USA), des simulations Monte Carlo ont été exécutées afin de calculer la probabilité d'infection annuelle associée à la transmission des

(oo)cystes de *Cryptosporidium* et *Giardia* internalisés dans l'eau potable. Le calcul de la probabilité annuelle d'infection est basé sur l'équation suivante :

$$P_{\inf(annual)} = 1 - (\exp(-r \times C_{IP\_Eff} \times V_d \times 365)$$

où r est un paramètre d'infectivité décrivant l'interaction hôte-pathogène, V_d est le volume d'eau moyen consommé quotidiennement et C_{IP_Eff} est la concentration des (oo)cystes internalisés dans l'effluent des filtres granulaires.

Une chaîne d'événements conduisant à la présence d'(oo)cystes internalisés dans l'effluent des filtres granulaires a été posée à la base du modèle développé, C_{IP_Eff} étant exprimée par l'équation suivante:

$$C_{IP\_Eff} = C_{Rot\_FB} \times N_{IP/Rot\_FB} \times S \times F$$

où C_{Rot_FB} = concentration des prédateurs (rotifères) dans les lit filtrant;

N_{IP/Rot_FB} = nombre d'(oo)cystes internalisés (IP) par rotifère dans le lit filtrant;

S = Probabilité pour un (oo)cyste internalisé de demeurer et survivre à l'intérieur de son hôte jusqu'à ce qu'il soit rejeté à l'effluent filtré;

F = Fraction des rotifères du lit filtrant rejetés à l'effluent filtré.

Le modèle conceptuel développé dans cette étude afin de décrire l'internalisation et le transport des (oo)cystes de *Cryptosporidium* et *Giardia* suite à la prédation par les rotifères dans un filtre granulaire est basé sur des simplifications de certains des processus complexes impliqués et encore peu caractérisés dans la littérature à ce jour : (i) la contamination du filtre et la rétention des (oo)cystes dans le lit filtrant ont été décrits comme un événement ponctuel en début de cycle de filtration; (ii) la prédation a été décrite comme une accumulation d'(oo)cystes internalisés à un taux constant à l'intérieur des prédateurs (rotifères), lesquels sont supposés présents en concentration constante dans le lit filtrant au cours d'un cycle de filtration; (iii) le transport des (oo)cystes internalisés à l'effluent est exprimé comme le produit du rejet des rotifères à l'effluent et du taux de persistance/survie des (oo)cystes internalisés dans un rotifère; (iv) la transmission des (oo)cystes internalisés dans l'eau potable est basée sur l'hypothèse d'une résistance complète

des (oo)cystes internalisés face aux traitements de désinfection subséquents, à l'exception de la désinfection UV.

La quantification de la plupart des variables impliquées dans le calcul du risque par le modèle développé a été dérivée de données générées à l'échelle pilote dans l'étude de filtration décrite à la section 2.2.1 de cette thèse et présentée en détails au chapitre 3. Des distributions ont été définies pour chacune des variables dans Crystal Ball® afin de représenter la variabilité et l'incertitude associées à la quantification de ces variables.

Une analyse de sensibilité a été effectuée à l'aide de Crystal Ball® afin d'évaluer l'impact des principales variables sur le risque d'infection calculé par le modèle.

CHAPITRE 3 PUBLICATION #2: ROLE OF PREDATION BY ZOOPLANKTON IN TRANSPORT AND FATE OF PROTOZOAN (OO)CYSTS IN GRANULAR ACTIVATED CARBON FILTRATION

Ce chapitre présente le développement d'une méthode expérimentale permettant de détecter et énumérer des (oo)cystes de *Cryptosporidium* et *Giardia* suite à leur internalisation par des organismes du zooplancton dans un filtre granulaire et dans son effluent. Les protocoles développés et optimisés dans cette étude ont été appliqués à des échantillons de zooplancton isolés du lit filtrant et de l'effluent de colonnes de filtration au charbon actif granulaire (CAG) à l'échelle pilote. Ces travaux ont été publiés dans *Water Research*.

ROLE OF PREDATION BY ZOOPLANKTON IN TRANSPORT AND FATE OF PROTOZOAN (OO)CYSTS IN GRANULAR ACTIVATED CARBON FILTRATION

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Abstract: The significance of zooplankton in the transport and fate of pathogenic organisms in drinking water is poorly understood, although many hints of the role of predation in the persistence of microorganisms through water treatment processes can be found in literature. The objective of this study was to assess the impact of predation by natural zooplankton on the transport and fate of protozoan (oo)cysts in granular activated carbon (GAC) filtration process. UV-irradiated unlabelled Cryptosporidium parvum and Giardia lamblia (oo)cysts were seeded into two pilot-scale GAC filtration columns operated under full-scale conditions. In a two-week period after seeding, a reduction of free (oo)cysts retained in the filter bed was observed. Zooplankton was isolated from the filter bed and effluent water on a 30 µm net before and during the two-week period after seeding; it was enumerated and identified. Rotifers, which are potential predators of (oo)cysts, accounted for the major part of the isolated zooplankton. Analytical methods were developed to detect (oo)cysts internalized in natural zooplankton isolated from the filter bed and effluent water. Sample sonication was optimized to disrupt zooplankton organisms and release internalized microorganisms. (Oo)cysts released from zooplankton after sonication were isolated by IMS and stained (EasyStainTM) for microscopic counting. Both Cryptosporidium and Giardia (oo)cysts were detected in association with zooplankton in the filter bed samples as well as in the effluent of GAC filters. The results of this study suggest that predation by zooplankton can play a role in the remobilization of persistent pathogens such as Cryptosporidium and Giardia (oo)cysts retained in GAC filter beds, and consequently in the transmission of these pathogens in drinking water.

Keywords: *Cryptosporidium*, GAC filtration, *Giardia*, predation, transport, zooplankton

3.1 Introduction

The significance of higher organisms in drinking water is attracting increased scientific attention as we are starting to better understand their capacity to vector waterborne pathogens. Predation on pathogenic organisms by zooplankton was observed in laboratory co-cultures; however the study of waterborne pathogens associated with zooplankton in field conditions remains limited to this day. Protists, namely amoebae, are being increasingly described as the Trojan horse of

microorganisms; their role as vectors of waterborne pathogens through water systems is becoming more accurately described and highlighted as a potential threat to water safety (Barker and Brown 1994, Loret et al. 2008). Meanwhile, in some specific cases, the detection of zooplankton in treated water can be paired with an unpredicted persistence of natural indicator bacteria through treatment processes or in distribution systems. For example, the persistence of total coliform bacteria in a distribution system was associated to their transport inside the gut of naturally occurring nematodes, which provided protection to internal bacteria against residual free chlorine (Locas et al. 2007). Such situation raises hypotheses about the role of predation and transport by zooplankton organisms in the exposure to internalized microorganisms in drinking water (Bichai et al. 2008).

Persistent organisms such as *Cryptosporidium* and *Giardia* (oo)cysts have been shown to be internalized by zooplankton in laboratory feeding experiments or co-cultures: *Cryptosporidium* oocysts have been observed within metanauplii of the microcrustacean *Artemia franciscana* (Mendez-Hermida et al. 2007), *Acanthamoeba* sp. subcultured from environmental water isolates (Gómez-Couso et al. 2007), *Acanthamoeba culbertsoni* and ciliates (Stott et al. 2003), as well as nematode *Caenorhabditis elegans* (Huamanchay et al. 2004). Various species of rotifers have been shown to ingest *Cryptosporidium* oocysts (Fayer et al. 2000) and *Giardia* cysts (Trout et al. 2002) in laboratory conditions. Ingestion of both of these protozoan (oo)cysts has also been reported with the cladoceran *Daphnia pulicaria* (Connelly et al. 2007). These studies were all based on direct microscopic methods to visualize the internalized (oo)cysts in pure predator/prey co-cultures. However, rarely have natural environmental zooplankton samples been examined for the recovery of internalized (oo)cysts: *Cryptosporidium* has been detected within rotifers in lake waters (Nowosad et al. 2007) by disrupting the animals before performing FISH detection. To our knowledge, no study has been conducted to date with the objective to detect *Giardia* cysts in environmental zooplankton samples.

The occurrence of higher organisms has been characterized in distribution systems (van Lieverloo et al. 2004) and at some stages of full-scale water treatment plants, mostly in granular material filter effluents (Schreiber et al. 1997, Castaldelli et al. 2005). Characterizing the

occurrence of waterborne pathogens and bacterial indicators internalized by zooplankton in field conditions presents important methodological challenges, and was to date only reported by King et al. (1991) and Nowosad et al. (2007) in surface waters, and by Wolmarans et al. (2005) and Thomas et al. (2008) in water treatment plants, the later focusing on amoebal hosts. Yet, the occurrence of internalized (oo)cysts in natural zooplankton samples from drinking water treatment processes has never been assessed. It is not known to which extent predation on (oo)cysts can impact the performance of full-scale drinking water treatment; yet, internalization by zooplankton has been speculated as a possible mechanism involved in the mass reduction of oocysts in slow sand filters seeded with UV-irradiated unstained oocysts of *C. parvum* (Hijnen et al. 2007).

Considering the naturally low concentrations of these organisms in water treatment plants, we propose that the most appropriate way to find evidence for the hypothesized mechanisms of transport of pathogens associated to zooplankton is to monitor environmental zooplankton under controlled conditions of seeding (oo)cysts at pilot-scale. The current study was performed additionally to the study presented by Hijnen et al. (this issue), in which two pilot-scale granular activated carbon (GAC) filters operated under full-scale conditions (natural water and filtration conditions),were seeded with UV-irradiated unlabelled (oo)cysts of *Cryptosporidium parvum* and *Giardia lamblia* to establish the elimination capacity for protozoan (oo)cysts. The objective of the present study was to investigate the fate of the retained (oo)cysts and the presence of (oo)cysts internalized by the natural zooplankton present in the GAC filter bed and filtrate. The mass reduction of (oo)cysts in the GAC filter bed during an extended filtration period of two weeks was determined. Simultaneously, zooplankton was isolated from the GAC material and effluent water, and an analytical protocol was developed for both types of samples to be examined for the occurrence of internalized (oo)cysts. To our knowledge, this is the first study to develop and apply a procedure for sample analysis specifically designed to detect both *Cryptosporidium* and *Giardia* (oo)cysts inside a natural population of zooplankton organisms, in an attempt to characterize the role of zooplankton in the ingestion and transport of (oo)cysts through a granular material filter bed and into the effluent water.

3.2 Material and Methods

3.2.1 GAC filtration seeding test

A pilot plant with two parallel GAC filter columns (15 cm diameter; 1 m deep, 5 $m.h^{-1}$ and contact time of 12 minutes; no back washing) were seeded for 2 hours at an influent concentration of ~$1.6x10^{5}.l^{-1}$ and $4.8x10^{4}.l^{-1}$ of UV-inactivated (unlabelled) *C. parvum* and *G. lamblia* (oo)cysts, respectively. The GAC originated from full-scale filters operated for 40 000 bed volumes of filtering. The complete description of the filtration set-up and seeding experiment is detailed in Hijnen et al. (this issue). This seeding test was conducted under full-scale conditions using the filtrate of a conventional treatment (coagulation and rapid sand filtration) supplied with the River Meuse water (the Netherlands) after storage in impoundment reservoirs.

3.2.2 Experimental protocol

The present study can be described in 3 main experimental parts: (i) To determine the occurrence of a mass reduction over time of the retained (oo)cysts in the filter bed as an hypothesized indication of the presence and activity of predators, GAC samples grabbed at various depths through the filter bed of both columns were analyzed one and three weeks after the seeding test. (ii) Natural zooplankton samples from slow sand filters and from the GAC pilot filters was used to develop the optimal protocol for complete disruption of the zooplankton in order to release and enumerate the internalized (oo)cysts. (iii) Natural zooplankton from the GAC material sampled three weeks after seeding and from the effluent of the GAC columns sampled one and three weeks after the seeding experiment was isolated, identified and quantified, and zooplankton was treated using the optimized disruption procedure in order to find evidence for the presence of internalized (oo)cyts. The procedures are summarized in **Figure 3.1**. Because of the use of unlabelled (oo)cysts in the seeding experiment direct microscopic evidence of internalized (oo)cysts in the environmental zooplankton could not be obtained in the scope of the current study.

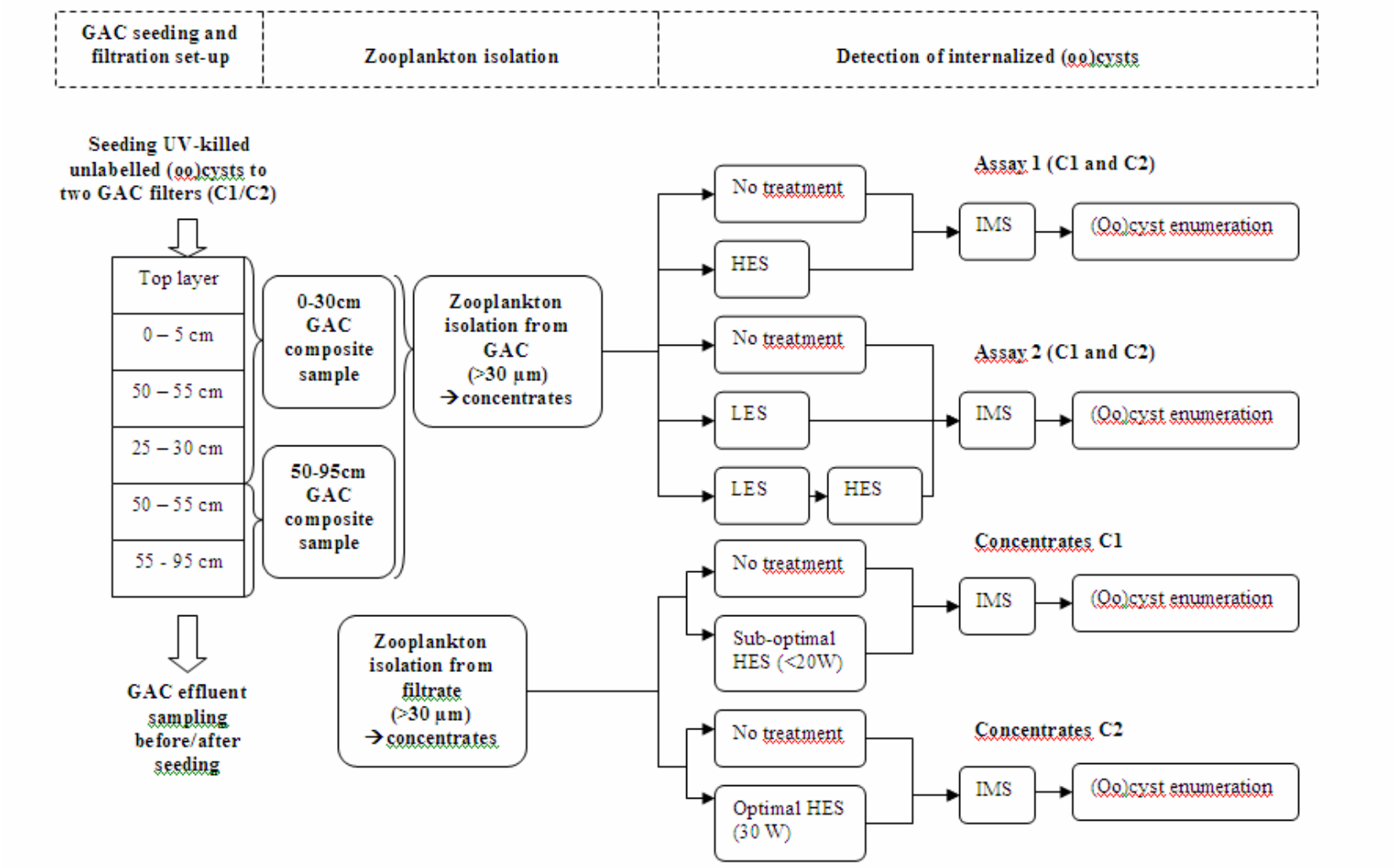


Figure 3.1: Experimental set-up and optimized detection procedures for internalized (oo)cysts in GAC material and effluent water isolated zooplankton

3.2.3 Analysis of free (oo)cysts retained in the GAC filter bed

One and three weeks after seeding (oo)cysts in the loaded GAC filters, GAC bulk samples were collected throughout the filter bed of both filtration columns according to the following distribution: top layer, 0-5 cm, 5-10 cm, 25-30 cm, 50-55 cm and bottom layer (85-90 cm) The bulk GAC samples were kept in closed jars at 4°C. The GAC sub-samples from every layer of the columns sampled one week after the seeding test were analyzed separately to measure the number of retained (oo)cysts. From the GAC sub samples taken three weeks after the seeding experiment two composite samples were prepared from the first four layers (0-30 cm) and last two layers (50-90 cm). The GAC sub-samples (5 g) were treated in separate suspensions of 20 ml autoclaved tap water with successively 2 minutes of hand shaking, 2 minutes of vortex mixing, 2 minutes of low energy sonication (LES) and 2 minutes of high energy sonication (HES) at 45% amplitude. The resulting sub-samples were combined into one suspension for (oo)cyst analysis (Hijnen et al., 2009, this issue).

3.2.4 Isolation of zooplankton from the GAC material

The GAC bulk samples from column 1 and 2 which were used for analysis of the mass reduction were also analyzed to identify and quantify the zooplankton and subsequently to determine the number of zooplankton-associated (oo)cysts. Two composite GAC sub-samples were made for each filtration column by weighting (in 80 ml of sterile tap water i.e. wet weight) 10-g GAC of each filter bed fraction from depth ≤30 cm (top layer, 0-5 cm, 5-10 cm, and 25-30 cm depth) and ≥50 cm (50-55 cm depth and bottom layer). Composite samples were washed 5 times by vigorous shaking with sterile tap water. All of the washing water was collected and filtered through a 30-µm net to retain the zooplankton. The recovered zooplankton sample was vortexed, filtered 3 additional times and thoroughly rinsed with sterile tap water through the 30-µm net in order to get rid of most free (oo)cysts in the sample and to concentrate the zooplankton.

3.2.5 Isolation of zooplankton from effluent water

Zooplankton was collected at the outlet of each of two replicate filtration columns using 30-µm plankton net before and after the seeding experiment. Two effluent volumes of 2100 and 2200 l were filtered through a 30-µm plankton net during a 24-hour period one week and 2 days prior to

seeding. Then a first sample of 6600-6800 l was collected two days after spiking over a 70-hr period, followed by a second volume of 2560 l collected 3 weeks after spiking over a 64-hr period. All samples were fixated in 4% formaldehyde and kept at 4oC until processed and analyzed for (oo)cyst recovery and zooplankton species identification and quantification.

3.2.6 Zooplankton sample preparation for method development

Natural zooplankton was used to develop the analytical protocol for recovering internalized (oo)cysts. Some fractions of the zooplankton concentrates isolated from the effluent water of the GAC filtration columns prior to the seeding test (unseeded zooplankton) as well as zooplankton samples isolated from the Schmutzdecke of slow sand filters were used in order to conduct preliminary tests to determine the optimal sonication conditions to disrupt the animals and extract internalized (oo)cysts. The Schmutzdecke from the slow sand filters was scraped from the top 2-3 cm layer of Weesperkarspel pilot-plant (Amsterdam, the Netherlands) while the filter bed was completely saturated with water. The filters had been in operation for several weeks without surface scraping. Subsequently, sand sub-samples were thoroughly washed in sterile (non-chlorinated) tap water 5 times and the rinsing water was filtered through a 30 µm net to collect the zooplankton organisms. Sub-samples of concentrated zooplankton suspensions prepared from slow sand filter samples or GAC effluent water samples (as described above) were put into Petri dish (9-mm diameter) and observed under a binocular (30X magnitude). Animals were picked up individually by using a pipette set to a volume of 5 µl and split into 3 groups of organisms with an expected similar resistance to sonication treatment: animals (in 5-µl drop) were then added to 5 ml of sterile tap water in a 15-ml centrifugation tube until the tube contained 20 organisms of one pre-determined group. The following groups were defined on the basis of a hypothesized difference in resistance to sonication treatment deduced from the presence/absence of a hard shell: (1) nematodes, (2) rotifers with a shell (Lecane sp., Colurella sp., others), and (3) rotifers without a shell (Philodina sp., Rotaria sp., others).

3.2.7 Zooplankton disruption procedure to extract internalized (oo)cysts

High energy sonication (HES) was used to extract the internalized (oo)cysts from the zooplankton. A Branson Sonifier S-250D was used at amplitudes of 45% and 65%, considering that the manufacturer recommends not using amplitude higher than 70% to avoid stress cracking

of the microtip. The performance indication of the apparatus for 5-ml samples in 15-ml centrifugation tubes was monitored, and the corresponding power output (W) was determined by using the Sonifier output control curves (Branson Sonifier S-250D, USA). Different sonication times were tested on a standardized volume (5-ml samples) in a standardized container (15-ml centrifugation tubes) kept on ice during the procedure to prevent temperature rise. To test the effect of this HES disruption procedure on the oocyst recovery and staining by *EasyStain*^{TM} (BTF), a suspension of UV-inactivated *C. parvum* oocysts (Waterborne^{TM}, ~3x10^{4} oocysts.ml^{-1}, in distilled water) was stained either prior to or after sonication (HES). Oocysts were counted on duplicate *Dynal*^{TM} slides using 50 µl volumes of the suspensions (stained/unstained) before and after sonication treatment.

3.2.8 Elimination of free (oo)cysts from the zooplankton concentrates and recovery tests

In order to focus the analysis on zooplankton-internalized (oo)cysts in this research, efforts were made to exclude free (non-internalized) (oo)cysts possibly present in the zooplankton samples. Two strategies were tested to attempt separating free (oo)cysts from zooplankton: (1) centrifugation and (2) sedimentation. For both methods, a *ColorSeed*^{TM} (BTF, Australia) sample (1 ml, 100 *Cryptosporidium* oocysts and 100 *Giardia* cysts) was added to 15 ml of unseeded effluent zooplankton samples from column 2. Centrifugation was applied at 500*g* for 2 minutes. Sedimentation was applied at 4^{o}C for 24h. After either separating procedure, the bottom part (pellet) of the sample (where most zooplankton was expected to be found) was divided in 2 fractions, one of which was treated with optimized zooplankton disruption treatment (HES, 40 s at 65% amplitude) and the other remained intact. Then, all fractions of the sample i.e. (i) upper part (supernatant), (ii) lower part sonicated, and (iii) lower part untreated were processed with the IMS method. Positive control slides were also analyzed for staining quality control. Recovery of the *ColorSeed*^{TM} in those tests was assessed in order to determine the recovery associated to the detection method used for (oo)cyst enumeration in the zooplankton samples (GAC material and GAC effluent) in this study.

3.2.9 Analysis of zooplankton concentrates from the GAC material

Analysis of zooplankton sub-samples for recovery of internalized (oo)cysts was performed twice (Fig. 1). The first time, the analysis allowed to compare (oo)cyst recovery from an untreated fraction of zooplankton concentrates with the (oo)cyst recovery after applying the optimized HES disruption procedure (Assay 1). The second time, a low energy sonication step (LES) was introduced in the handling of the zooplankton isolates in order to detach free (non-internalized) (oo)cysts which from carbon particles observed in the washed zooplankton concentrates (Assay 2). Performing a LES treatment for 2 minutes in an ultrasonic bath (Branson Model 5510, Danbury, USA) has previously shown to efficiently release (oo)cysts attached to carbon fines in GAC material samples (Hijnen et al. 2009, this issue), while not disrupting zooplankton (data not shown). In Assay 1, each of the zooplankton concentrates from GAC composite samples was mixed and divided into 2 sub-samples: (i) 10-ml was untreated; (ii) 10 ml was sonicated (HES) on ice (in 5-ml sub-samples, 40 seconds at 65% amplitude); in Assay 2, zooplankton concentrates mixed and split into 3 fractions: (i) 10 ml was kept untreated; (ii) 10 ml was treated with LES for 2 minutes in glass tubes to detach (oo)cysts associated to carbon fines; and (iii) 10 ml was first treated with LES for 2 minutes, followed by HES disruption procedure (on ice, in 5-ml sub-samples, 40 seconds at 65% amplitude).

3.2.10 Analysis of zooplankton concentrates from the effluent water

Effluent samples from column 1 and column 2 were analyzed for the detection of zooplankton-associated (oo)cysts. Samples from column 1 were all analyzed concomitantly with the method development. All effluent zooplankton samples from column 1 (pre- and post-seeding) were mixed and split into 5-ml volumes, and sonicated on ice for 40 seconds at 65% amplitude in 50-ml plastic tubes. The sonicated suspension was transferred in a *Dynal*^{TM} tube for further IMS processing. A positive control slide was included as staining quality control. Pre-seeding samples from column 2 were also analyzed throughout the method development phase (see recovery tests described in a previous section). Post-seeding samples from column 2 were analyzed with the optimized procedure (without any additional step to separate free (oo)cysts from internalized ones). The entire post-seeding samples from column 2 were homogenized (vortex) and divided into several 5-ml sub-samples which were sonicated on ice (40 s, 65% amplitude) to disrupt the zooplankton organisms. Finally, the samples were processed with the

IMS detection method and further analyzed by fluorescence microscopy for enumeration of recovered (oo)cysts.

3.2.11*Cryptosporidium*, *Giardia* and zooplankton enumeration

In the treated or untreated sub-samples, *Cryptosporidium* and *Giardia* (oo)cysts were determined by IMS and counted with epifluorescence microscopy (Leica, DM RXA) at a 250X magnitude as previously described (Hijnen et al., 2007). The concentration of internalized (oo)cysts in the GAC material isolated from the zooplankton samples was corrected for the recovery of the analysis as assessed in the current study and expressed in $n.ml^{-1}$ using the volume weight of the GAC of 0.66 $g.ml^{-1}$.

For zooplankton enumeration in the GAC material and the GAC effluent, samples of 1 ml to 5 ml of zooplankton concentrate were transferred into a counting plate, allowed to settle for 5 minutes. The entire counting chamber was scanned and organisms were enumerated with an inverted microscope (Leica, Leitz Labovert FS) (100X magnitude).

3.3 Results and discussion

3.3.1 Analysis of free (oo)cysts retained in the GAC filter bed.

The concentration of retained (oo)cysts of *C. parvum* and *G. lamblia* in the GAC filters sampled one week and three weeks after the seeding experiment are presented in **Figure 3.2**. An average mass reduction of *Cryptosporidium* oocysts of 66.2% (66.1% – 66.3%) and 32.1% (-4.6% – 68.2%) was found in the upper and lower parts of the GAC filter beds, respectively. This mass reduction of retained oocysts is for a minor part (<5%; calculated from water flux and effluent concentrations) due to wash out, and therefore mostly attributed to processes in the filter bed, such as predation. Due to the large variation in *G. lamblia* cyst concentration determined one week after the seeding, the slight mass reduction was not significant for this microorganism.

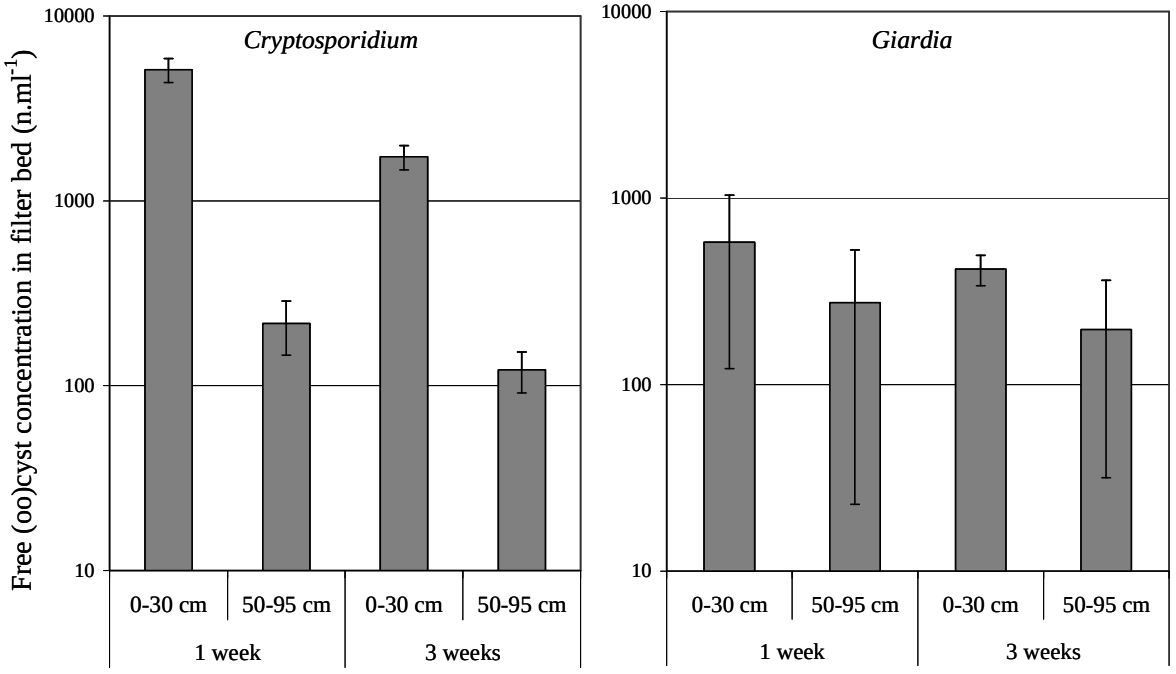
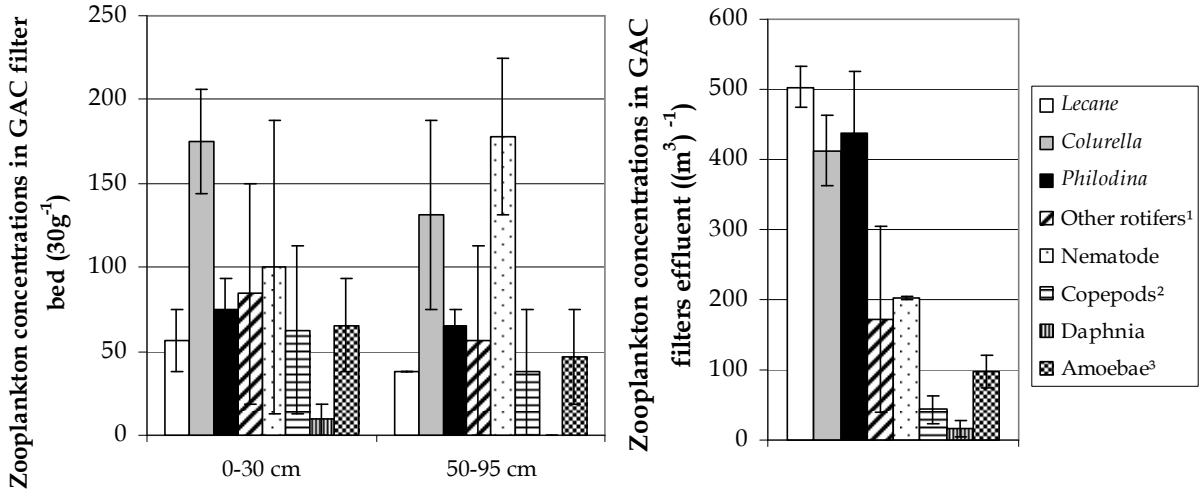


Figure 3.2: Concentrations of free (a) *Cryptosporidium* and (b) *Giardia* (oo)cysts retained in the GAC filter bed 1 and 3 weeks after the seeding test (error bars show the range of data).

3.3.2 Zooplankton enumeration.

Figure 3.3 shows the average concentration of zooplankton organisms found in the effluent of the GAC columns and in the GAC material sampled from the columns. Concentrations of >300 and >800 organisms/30 g of GAC material were estimated in the filter bed of column 1 and 2, respectively. In the effluent water, concentrations of ~1600 organisms/m^3 and ~2100 organisms/m^3 were calculated for columns 1 and 2, respectively. Although these data are based on limited sample analysis, they indicate a higher zooplankton density in column 2 as compared to column 1. In both columns, zooplankton was found to be distributed throughout the whole depth of the filter bed. Rotifers (mostly *Lecane*, *Philodina* and *Colurella* spp.) were found to be

the dominant zooplankton population, followed by nematodes, both in GAC material and effluent water samples. In both columns, rotifers and nematodes were observed to account respectively for ~80% and ~10% of the total number of zooplankton organisms released in effluent waters. Since rotifers are known to internalize (oo)cysts both in laboratory conditions (Fayer et al. 2000, Trout et al. 2002) and in natural aqueous environment (Nowosad et al. 2007), they are suspected to be potential predators of (oo)cysts in granular filters. The important proportion of rotifers observed in the zooplankton populations rejected in the GAC filters effluent suggests that they could also have a role of transport of internalized (oo)cysts into filtered water.



(a) (b)

Figure 3.3: Average concentrations of zooplankton organisms (error bars show the range) from column 1 and 2 (a) in the GAC material ($30g^{-1}$) and (b) in the effluent water ($(m^3)^{-1}$) in upper and lower parts of the filter bed. (1*Brachionus, Trichocerca, Bosmina, Polyarthra, Asplancha, Testudinella;* 2*Harpaticoida*, nauplii, others; 3*Testacea, Euglypha*)

3.3.3 Zooplankton disruption procedure to extract internalized (oo)cysts.

Sonication tests using HES were conducted with the objective to find the optimal conditions (intensity and time) that would result in a complete (and if possible simultaneous) disruption of all zooplankton organisms present in the concentrated samples to release internalized (oo)cysts.

Results obtained for increasing sonication durations at amplitudes of 45% or 65% are shown in **Table 3.1**. The efficacy of the treatment was calculated as: $(n_{before\_HES} - n_{after\_HES})/n_{before\_HES}$ x100%, with n_{before_HES}= 20 zooplankton organisms for all suspensions and all HES conditions tested. The disruption was more effective at a 65% amplitude with a resulting power output of 30 W than at 45% with a power output of 15-20 W, and the higher power output allowed a better synchronization of the disruption of the various zooplankton species present in the samples. After 40 seconds at 65% amplitude, ≥95% of all tested classes of zooplankton were disrupted. These conditions were selected as the optimized disruption procedure for further treatment of the GAC zooplankton samples. The selected HES treatment was applied to a suspension of *C. parvum* (oo)cysts (in the absence of zooplankton) to determine its impact on the recovery and the staining of free (oo)cysts following IMS. Average recovery rates calculated from: $(n_{before\_HES} - n_{after\_HES})/n_{before\_HES}$ x100% (n = number of oocysts) were 42% (± 1%; n = 2) for oocysts stained prior to HES and 86% (± 15%; n = 2) for oocysts stained after HES. These results suggest that HES at 65% amplitude (30 W) for 40 seconds does have a significant impact on the recovery of pre-stained *Cryptosporidium* oocysts. During microscopy enumeration, some oocysts were observed to be damaged due to sonication. However, the recovery of unstained oocysts was not statistically different from 100% (p =0.63), which reproduces the condition of the oocysts seeded in the GAC filtration experiment. This test also allowed concluding that the staining of oocysts by *EasyStain*^{TM} was not, in itself, negatively influenced by sonication. Considering (i) the low impact of the HES disruption on the unstained UV-inactivated oocysts and (ii) the assumption that the internalized (oo)cysts are mostly protected during the HES disruption until they are released from zooplankton hosts, it was concluded that the HES disruption procedure used to release internalized (oo)cysts had a negligible effect on the enumeration of these (oo)cysts.

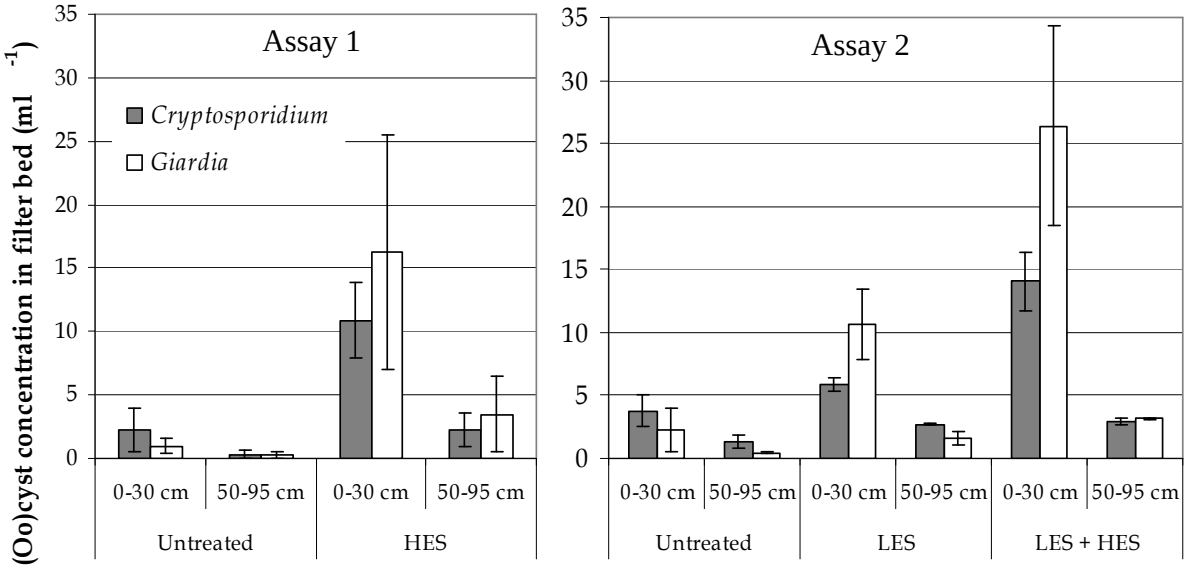
Tableau 3.1: Sonication (HES) treatment efficiency for zooplankton disruption

Amplitude	**45%**		**65%**				
Performance indication	10-15 %		20%				
Power delivered	15-20 W		30 W				
Sonication time (s)	**60**	**90**	**20**	**25**	**30**	**35**	**40**
Nematodes	70	95	90	100	100	100	*nd*
Rotifers with shell	90	90	*nd*	*nd*	90	nd	100
Rotifers without shell	85	100	70	80	*nd*	95	95
Harpaticoida	*nd*	*nd*	*nd*	*nd*	*nd*	*nd*	100

nd: not determined

3.3.4 Analysis of zooplankton concentrates from the GAC material.

In the GAC material, free (non-internalized) (oo)cysts are present, as was demonstrated in **Figure 3.2**. Following the zooplankton isolation procedure from the GAC material, part of these free (oo)cysts can remain present in the zooplankton concentrates. Therefore, (oo)cysts in these zooplankton samples were enumerated with and without the HES disruption procedure. The results clearly showed the presence of free (oo)cysts (Fig. 3.4; untreated samples). In both Assay 1 and 2, after HES disruption of the zooplankton samples, the number of (oo)cysts increased significantly (Fig. 3.4a,b), especially in the samples from the upper part of the filter. The estimated zooplankton density in this part of the filter bed was also slightly higher for most organisms (Fig. 3.3). In Assay 2, an additional LES treatment was applied to enumerate (oo)cysts retained on GAC fines present as part of the pellet in these zooplankton samples. Preliminary tests showed that low energy sonication (LES) had no effect on zooplankton organisms when performed for up to 10 minutes (data not shown). The results showed an increase of (oo)cysts (Fig. 3.4b) after LES, yet the number of (oo)cysts enumerated after the zooplankton disruption procedure (HES) increased again significantly. These data can be regarded as strong indications for the presence of internalized (oo)cysts from *C. parvum* and *G. lamblia* associated to zooplankton organisms.



(a) (b)

Figure 3.4: Average concentrations of free and internalized (oo)cysts (error bars show the range) from column 1 and 2 in upper and lower parts of the filter bed (ml^{-1}) assuming 100% recovery in (a) Assay 1 and (b) Assay 2. (*LES: Low energy sonication, HES: High energy sonication*)

3.3.5 Analysis of zooplankton concentrates from the effluent water.

The presence of free (oo)cysts in the zooplankton concentrates collected from the carbon filter effluents by filtration on a 30-µm plankton net is not very likely. However, if some free (oo)cysts were to be retained in the 30-µm plankton net when isolating zooplankton, those could not be distinguished from the (oo)cysts extracted from zooplankton. To account for that possibility, although it is thought to be of low probability, two methods were tested to attempt eliminating free (oo)cysts from the zooplankton samples before performing the disruption procedures: centrifugation and sedimentation, both tested by spiking *ColorSeed*^{TM} in unseeded effluent zooplankton samples from column 2. The results of these tests (not presented) showed that separation was not achievable: both (oo)cysts and zooplankton organisms were present in the

pellet and the supernatant after both separation procedures. Therefore, for all further analyses, no separation step was applied on the zooplankton concentrates before the HES treatment.

Recovery rates were calculated through those two tests by considering the total recovery of *ColorSeed*^{TM} (oo)cysts (in pellet and supernatant) that had been spiked in the zooplankton concentrates. Recovery rates of 61% and 15% for *Cryptosporidium* oocysts and *Giardia* cysts, respectively, were calculated and were used for correcting the concentrations of internalized (oo)cysts in the filter bed and effluent of GAC columns when interpreting our results. These calculated recovery rates in zooplankton concentrates were higher than those observed for recovery of free (oo)cysts in the GAC effluent during the filtration study by Hijnen et al. (this issue), which were determined as 13% for *Cryptosporidium* and 7% for *Giardia*. Those rates were used to correct free (oo)cyst concentrations in the interpretation of our results.

A marked difference was observed in the results of the analyses of zooplankton samples from effluent water of column 1 and 2, which can be explained by the differences in the procedure through which they were handled. Zooplankton concentrates from effluent water of column 1 were analyzed throughout the phase of methodology development and were all found to be negative for the presence of *Cryptosporidium* and *Giardia* (oo) cysts, except for a post-seeding sample in which one *Cryptosporidium* oocyst was found. However, the HES disruption procedure applied on effluent samples from column 1 was different from the optimized procedure subsequently applied to all the other zooplankton concentrates: the HES disruption procedure in the 5 ml subsamples was performed in larger 50-ml centrifugation tubes instead of 15-ml tubes, and the sonifier indicated a low performance (~10%) which corresponds to a power of <20 W being transmitted to the sample. This impacted the disruption efficacy, which was confirmed by the microscopic observation of some intact animals in the treated samples. For all subsequent zooplankton concentrate analyses (all GAC material concentrates from both columns and effluent from column 2), 15-ml centrifugation tubes were used, in which the surface area of the 5-ml concentrate exposed to standard HES sonication is reduced, therefore yielding a higher sonication power.

Zooplankton concentrates from effluent water of column 2 were treated with this optimized HES disruption procedure (40 s at 65% amplitude, >30-35 W). In these disrupted concentrates no

intact zooplankton organisms were found, but they did contain numerous (2-125) (oo)cysts (**Table 3.2**). The number of (oo)cysts in the zooplankton concentrates collected three weeks after the seeding of (oo)cysts in the GAC filters was terminated were higher than in the concentrates sampled one week after seeding, even though a smaller volume of water was filtered (2.0 vs 6.6 m^3, see **Table 3.2**). This is an indication that the detected (oo)cysts had been transported inside of zooplankton organisms rather than free in the effluent water, since free (oo)cyst concentrations would normally have been expected to decrease through time after the seeding was stopped. The increase of the number of internalized (oo)cyst in the filtrate may indicate either a retarded breakthrough of internalized (oo)cysts or an increased grazing efficiency of zooplankton in the filter bed over time. Furthermore, the fact that higher sonication conditions in the analysis of effluent samples from column 2 allowed detecting (oo)cysts as opposed to samples of column 1 (treated with sub-optimal HES procedure) reinforces the demonstration that recovered (oo)cysts from column 2 were in fact internalized and efficiently extracted from zooplankton organisms through the optimized protocol elaborated in this study. Detection of internalized (oo)cyst from effluent water samples is an indication that predation by zooplankton can favor persistence of (oo)cysts in filter beds and act as a vehicle for (oo)cysts to be released into filtered effluents. Although further confirmation is required, to our knowledge these are the first observations that strongly indicate that predation by natural zooplankton can favor persistence of *Crytosporidium* and *Giardia* (oo)cysts in GAC filter beds and act as a vehicle for (oo)cysts to be released into filtered effluents.

Tableau 3.2: Internalized (oo)cysts in zooplankton concentrates and free (oo)cysts from effluent water sampled one week and 3 weeks after the seeding test

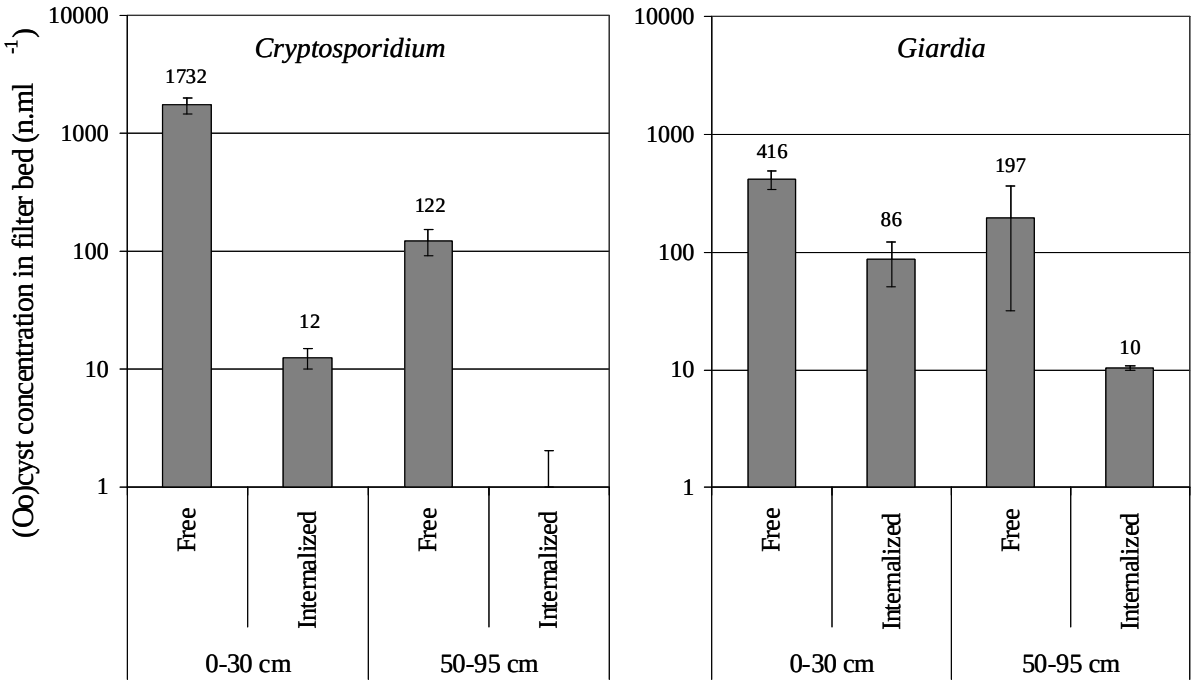
Column/time after seeding		Sampled effluent volume (l)	Internalized (oo)cysts in zooplankton effluent concentrates			Free (oo)cysts in effluent water	
			C. parvum n; C ($n.l^{-1}$)[b]	*G. lamblia* n; C ($n.l^{-1}$)[b]	Rotifers (n)[c]	*C. parvum* C ($n.l^{-1}$)[d]	*G. lamblia* C ($n.l^{-1}$)[d]
C1[a]	Week 1	6700	1; 0.0002	0; <0.001	8900	19.3	0.09
	Week 3	2000	0; <0.0008	0; <0.004	2700	nd	nd
C2	Week 1	6700	9; 0.002	2; 0.002	11400	17	0.5
	Week 3	2000	125; 0.102	64; 0.246	3500	nd	nd

[a] Sub-optimal HES disruption (Fig. 1); [b] Concentrations corrected for recovery of 61 and 13% for *Cryptosporidium* and *Giardia*; [c] Estimated numbers from enumeration data presented in Fig. 3b; [d] Corrected for the recovery of the analysis of free (oo)cysts (Hijnen et al., 2009); n = number; C = concentration; nd = not determined

3.3.6 Importance of the internalization of (oo)cysts by zooplankton in GAC filtration.

For each part of the filter bed of both columns in Assay 2, the concentration of internalized (oo)cysts was calculated by subtracting the (oo)cyst counts found after LES from the total number obtained after zooplankton disruption (LES + HES). Data from Assay 1 (without LES) were corrected using the results from Assay 2 to estimate the proportion of the raise in (oo)cyst counts following HES which is due to detachment from carbon particles. The average concentrations of internalized (oo)cysts in the upper and lower parts of the filter bed ($n.ml^{-1}$) from column 1 and 2 are shown in **Figure 3.5** (numbers corrected for recovery as presented before; 61% and 15 % for *Cryptosporidium* and *Giardia* (oo)cysts, respectively) and compared with the concentrations of free (oo)cysts 3 weeks after seeding (data from **Figure 3.2**). From these numbers, we calculate that less than 1% of *Cryptosporidium* oocysts in the filter bed were internalized. For *Giardia*, a higher percentage was estimated (17% in the upper part of the filter bed and 5% in the lower part) but these data are less reliable due to a higher variability in the measured concentrations of free cysts (see Fig. 2). These percentages are based on average values from both columns; when considered separately, higher proportions of internalized (oo)cysts are found in column 2. This level of internalization is expected to be reflected in the amount of internalized (oo)cysts in the zooplankton isolates from the effluent of both columns. Estimated concentrations of internalized (oo)cysts in GAC filters effluent water are presented in

Table 2. Numbers are found to reach >100 *Cryptosporidium* oocysts/m^3 and >200 *Giardia* cysts/m^3 in column 2 (most densely populated with zooplankton) 3 weeks after the seeding test. Free (oo)cysts were not measured in the effluent water 3 weeks after seeding, but concentrations would be expected to decrease in the effluent as compared to concentrations measured 2 weeks earlier, when free *Giardia* cysts were found in a concentration of ~500 cysts/m^3. For effluent water samples, the percentages of internalized (oo)cysts were found to be ≤1% for internalized (oo)cysts and *Cryptosporidium* oocysts, respectively, in effluent water collected 1 week after spiking. However, if calculating the same ratio 3 weeks after seeding, *Giardia* cysts are found to account for nearly 30% of the total cysts in the effluent water of column 2. This calculation is based on the assumption that the concentration of free (oo)cyst in effluent water 3 weeks after seeding would be the same as 2 weeks earlier. This assumption is conservative: it underestimates the calculated ratio of internalized (oo)cysts, since free (oo)cysts can be expected to decrease in the effluent water 3 weeks after stopping the seeding. These results may be an indication that although the process of remobilization of persistent pathogens in filter beds by predation is possible, the level is most likely low as compared to concentrations of free (oo)cysts. Considering the very low targeted concentrations of (oo)cysts in treated water (<1/100 000 l, USEPA 2006), the assessment of the significance of internalization and transport of (oo)cysts by zooplankton for water safety would need further investigation, which could be undertaken with the perspective of performing a quantitative microbial risk analysis (QMRA).



(a) **(b)**

Figure 3.5: Average concentrations of free and internalized (oo)cysts of (a) *Cryptosporidium* and (b) *Giardia* (error bars show the range) from column 1 and 2 in upper and lower parts of the filter bed (ml^{-1}); concentrations were corrected for the assessed recovery percentages (61% and 15 % for internalized *Cryptosporidium* and *Giardia* (oo)cysts, respectively, and of 13% and 7% rates for free (oo)cysts of *Cryptosporidium* and *Giardia*, respectively).

The current experimental protocol (seeding study) represents a worst-case scenario in which predation on (oo)cysts by natural zooplankton was favored under defined conditions (high concentration of seeded (oo)cysts) in an environmental system. The viability and infectivity of those internalized (oo)cyst remains however undetermined. If (oo)cysts internalized by zooplankton were found to remain infectious, as was demonstrated in the case of the ingestion of *Cryptosporidium* oocysts by the nematode *C. ele*gans (Huamanchay et al. 2004), then predation could be seen as increasing the persistence and the transport of these pathogens through filters. However, if (oo)cysts are being degraded by their predators before being naturally rejected in the

filter bed or in the effluent water, then predation would be found to be an important mechanism in the removal of protozoan (oo)cysts in biological granular filtration. Connelly et al. (2007) reported a significant decrease in viability of *Giardia* cysts and infectivity of *Cryptosporidium* (oo)cysts due to grazing by the crustacean *Daphnia pulicaria* in lab-scale feeding experiments. On a final thought, our experimental protocol targeted zooplankton larger than 30 µm, therefore excluding the potential impact of most amoebae, while *Acanthamoeba* has been shown to be a potential vehicle for *Cryptosporidium* oocysts (Gómez-Couso et al. 2007). Also, the 30-µm might allow some nematodes to escape due to their small width. The recovery of the zooplankton isolation method is unknown. It is likely that not all zooplankton organisms were extracted from the GAC material samples.

3.4 Conclusions

Our results suggest that predation by zooplankton population in a GAC filter bed has an effect on the transport and fate of pathogenic protozoan (oo)cysts retained in these filter beds. In two GAC filter columns pre-seeded with UV irradiated unlabelled (oo)cysts of *C. parvum* and *G.lamblia*, a reduction was measured in the retained mass of (oo)cysts during two weeks of filtration. Zooplankton was observed in the GAC material (5-200 organisms per 30 g) and in the filtrates of both columns (10-500 organisms.m^{-3}) which consisted for a large part of rotifers. Rotifers are known to ingest *Cryptosporidium* and *Giardia* (oo)cysts under laboratory conditions, and they are thought to be the main potential predator species for (oo)cysts in this study. A high energy sonication (HES) treatment was designed to disrupt the observed zooplankton and to recover internalized *Cryptosporidium* and *Giardia* (oo)cysts. . (Oo)cyst recovery was enhanced in zooplankton concentrates from the GAC filter bed when disruption of zooplankton organisms was performed by this procedure. In the zooplankton isolates from the GAC filters effluents, an increasing number of (oo)cysts was detected when the proper HES disruption method was applied. These observations are indications for the occurrence of internalized (oo)cysts in the environmental zooplankton present in the GAC filter bed and effluent water. Under the seeding conditions of this study, the ratio of internalized (oo)cysts to the total (oo)cyst concentration (free and internalized) in the filter bed and effluent water were found to be limited. The observed increase in the number of internalized (oo)cysts in the zooplankton concentrates from the effluent 3 weeks after the seeding test can be seen as an indication of increased predation activity over

time. The significance of the present findings for the microbiological safety of drinking water requires further research. More quantitative information on predation and remobilization and on the fate of internalized pathogens (viability/infectivity) is required to assess the probability of transmission of internalized infectious (oo)cysts by zooplankton under real environmental conditions, in the perspective to calculate the level of risk arising from these internalized organisms in drinking water.

Acknowledgements: The authors acknowledge the Industrial-NSERC Chair in Drinking Water and its industrial partners, namely the City of Montreal, John Meunier Inc. and the City of Laval. The collaborative work with KWR Watercycle Research Institute was part of the joint research program of the Ducth Water Supply Companies. The support during the GAC filtration experiment and with the zooplankton sampling from Dr. G.M.H. Suylen (Water Supply Company Evides) and C. Hoogendorp (Aqualab) and during the laboratory work at KWR by Anke Brouwer-Hanzens is greatly appreciated.

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CHAPITRE 4 PUBLICATION #3: PROTECTION AGAINST UV DISINFECTION OF *E. COLI* BACTERIA AND *B. SUBTILIS* SPORES INGESTED BY *C. ELEGANS* NEMATODES

Ce chapitre présente des résultats d'essais d'inactivation UV visant l'évaluation de l'effet de protection dû à l'internalisation par des nématodes de microorganismes cibles, soit les bactéries *E. coli* et les spores de *B. subtilis*. Un protocole de sonication a été développé afin de récupérer les bactéries internalisées avant ou après l'exposition de nématodes au rayonnement UV. Ces travaux ont été publiés dans *Water Research*.

PROTECTION AGAINST UV DISINFECTION OF *E. COLI* BACTERIA AND *B. SUBTILIS* SPORES INGESTED BY *C. ELEGANS* NEMATODES

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Abstract: Nematodes, which occur abundantly in granular media filters of drinking water treatment plants and in distribution systems, can ingest and transport pathogenic bacteria and provide them protection against chemical disinfectants. However, protection against UV disinfection had not been investigated to date.

In this study, *C. elegans* nematodes (wild-type strain N2) were allowed to feed on *E. coli* OP50 and *B. subtilis* spores before being exposed to 5 and 40 mJ/cm^2 UV fluences, using a collimated

beam apparatus (LP, 254 nm). Sonication (15 W, 60 s) was used to extract bacteria from nematode guts following UV exposure in order to assess the amount of ingested bacteria that resisted the UV treatment using a standard culture method. Bacteria located inside the gut of *C. elegans* were shown to benefit from a significant protection against UV. Approximately 15% of the applied UV fluence of 40 mJ/cm^2 (as typically used in WTP) was found to reach the bacteria located inside nematode guts based on the inactivation of recovered bacteria (2.7 log reduction of *E. coli* bacteria and 0.7 log reduction of *B. subtilis* spores at 40 mJ/cm^2). To our knowledge, this study is the first demonstration of the protection effect of bacterial internalization by higher organisms against UV treatment, using the specific case of *E. coli* and *B. subtilis* spores ingested by *C. elegans*.

Keywords: *B. subtilis* spores, *C. elegans*, *E. coli*, nematode, UV disinfection, water

4.1 Introduction

Higher organisms can harbour viable microorganisms and protect them to some extent from drinking water disinfectants (Bichai, F. et al. (2008)). The replication of *Legionella sp.* inside amoebae is probably the most documented example of this phenomenon and one of the main reasons for increased scientific attention dedicated to the complex issue of biological systems in which pathogenic microorganisms closely interact with higher organisms. On the other hand, the ecology of higher organisms, such as zooplankton and nematodes, is poorly understood and rarely taken into account in the water treatment industry. Within the scope of reducing the microbial risk associated with drinking water by using a multi-barrier approach, a question is raised regarding the risk associated with the internalization of waterborne pathogens by higher organisms. Nematodes, which are abundant in surface waters (Mott, J.B. and Harrison, A.D. (1983), Lupi, E. et al. (1995)), in granular media filters of water treatment plants (Matsumoto, N. et al. (2002), Castaldelli, G. et al. (2005), Hijnen, W.A.M. et al. (2007)), and in distribution systems (van Lieverloo, J.H.M. et al. (2004), Locas, A. et al. (2007)), have been shown to ingest, harbour and transport pathogenic bacteria and protect them against chemical disinfectants, including chlorine, as reviewed by Bichai, F. et al. (2008). While UV treatment is widely

recognized as an innovative and effective process for reducing the microbial risk in drinking water ({Wright, Gaithuma, et al. 2006}), the protection offered to bacteria when internalized by higher organisms (including nematodes) against UV radiation has not yet been investigated. Consequently, this study aims to assess the protection against UV disinfection of *E. coli* bacteria and *B. subtilis* spores ingested by *C. elegans* nematodes.

4.2 Materials and Methods

A protocol was developed to (i) feed nematodes with two target organisms: *E. coli* and *B. subtilis* spores, (ii) irradiate the resulting co-suspension with low pressure UV lamps, and (iii) recover the internalized organisms using sonication. The following sections describe in detail this procedure.

4.2.1 Maintenance of *C. elegans*

The nematode *Caenorhabditis elegans* has been shown to ingest and vector a variety of human pathogens and is commonly used as a biological model to investigate host-pathogen interactions (Bichai, F. et al. (2008)). A wild type strain (N2) of *C. elegans* was used in this study. Worms were maintained on 5-mm NGM agar plates, which contain (per litre of pure water): sodium chloride (3.0 g), agar (17 g), and Bacto-tryptone (2.5 g), $CaCl_2$ (111 mg), 5 mg cholesterol in 1 ml ethanol, $MgSO_4$ (247 mg), and KPO_4 (3.35 g). *E. coli* OP50 expressing the green fluorescent protein (GFP) was cultured at 37 ^oC for 24 h in L broth, which contains (per litre of pure water): sodium chloride (5.0 g), Bacto-yeast (5.0 g), and Bacto-tryptone (10.0 g). *E. coli* OP50 is a non-pathogenic strain routinely used as a feed source for *C. elegans* (Steirnagle 2006). The NGM agar was surface inoculated with 0.1 ml of a 24-h culture of *E. coli* OP50 – GFP and incubated at 37^oC for 24 h to establish confluent growth before transferring the worms.

4.2.2 Synchronizing Nematode Cultures

Nematode cultures were kept at room temperature (20-22 ^oC). The surface of 6 NGM agar plates, each containing a dense population of living nematodes of all ages, including eggs, was

washed by depositing 5 ml of sterile Milli-Q water and gently rotating the plate to float off the nematodes. The suspended eggs and worms were aseptically transferred to a sterile 15-ml centrifuge tube. Eggs and worms were collected by centrifugation (500 g, 2 min). The supernatant was removed and the pellet was resuspended in 10 ml of sterile Milli-Q water. The suspension was centrifuged again (500 g, 2 min) and the pellet was resuspended in 7 ml of a freshly made solution containing 5 ml of 1N NaOH and 2 ml of commercial bleach (~5.25% sodium hypochlorite) and then incubated at 20 ^{o}C for 10 minutes, with brief and gentle vortexing every 2 minutes to resuspend settled worms. This procedure kills all life cycle forms of the worm except the eggs (Kenney, S.J. et al. (2004), Stiernagle 2006). The suspension was centrifuged and washed three more times with sterile Milli-Q water to obtain a final suspension of approximately ~0.1 ml containing several eggs. A 50 µl aliquot was deposited on the surface of an NGM agar plate on which a lawn of *E. coli* OP50 - GFP had formed, and the plate was incubated at room temperature for 3 days to obtain adult worms. Nematodes collected on the third day of incubation were used in all disinfection assays. Typically, six plates containing such synchronized worm cultures were needed to produce the worm suspension used in a UV disinfection assay.

4.2.3 Preparation for UV Inactivation Assays

For each bacterial type, the complete experimental protocol described hereafter was entirely replicated three times (during three successive weeks). Each replicate disinfection assay was performed using new synchronized worm cultures.

For *E. coli* inactivation assays, worms were collected from the synchronized culture plates on the third day without further manipulations since the food source was also the targeted internalized bacteria for UV inactivation. The surface of all plates was washed with 5 ml sterile phosphate buffer and the plates were rotated gently to float off the nematodes. The suspensions were transferred into one sterile 50-ml centrifuge tube and sterile phosphate buffer was added to yield a final volume of 50 ml.

For spore inactivation assays, worms were collected from synchronized culture plates and the contents from each plate were aseptically transferred into a sterile 15-ml centrifuge tube. Every suspension was then washed three times with phosphate buffer as described previously in order to remove most of the external (un-ingested) *E. coli* and favour subsequent ingestion of the spores by nematodes. The worm pellets obtained were resuspended in 5 ml of sterile phosphate buffer and 0.1 ml of a solution of *Bacillus subtilis* spores (ATCC 6633) containing ~10^8 CFU/ml was added to each suspension. Each nematode-spore suspension was transferred into a 50-mm sterile Petri dish and incubated at room temperature for 3 h, which was sufficient time to allow spore accumulation inside the nematode gut (Laaberki, M.-H. and Dworkin, J. (2008)). Spores are reported to be more resistant to digestion (Laaberki, M.-H. and Dworkin, J. (2008)) than *E. coli* bacteria. After feeding, the content of all Petri dishes was aseptically transferred into one sterile 50-ml centrifuge tube and sterile phosphate buffer was added to a final volume of 50 ml.

4.2.3.1 Worm Suspension Washing Procedure for Reducing Chlorine Demand

Prior to the disinfection assays, the prepared nematode-prey suspensions were always washed according to the following procedure in order to reduce chlorine demand. Chlorination, described later, was used to inactivate external organisms. For both *E. coli* and *B. subtilis* spore experiments, the 50-ml worm and bacteria suspension was vortexed for 10 seconds, then centrifuged (500 g, 2 min) and left at 4 ^{o}C for 20 minutes to allow settling of the worms. The supernatant was then carefully removed and the remaining 2-ml suspension was resuspended in 20 ml of sterile phosphate buffer. This entire procedure was repeated once more before adjusting the volume of the final suspension to 50 ml by adding sterile phosphate buffer. The obtained suspension was homogenized by gentle vortexing and then analyzed to measure the initial concentration of bacteria and nematodes.

4.2.4 UV Inactivation Assays

4.2.4.1 Chlorination

Suspensions were chlorinated using a 750 mg/l (as Cl_2) sodium hypochlorite solution prior to UV inactivation assays in order to surface-sterilize the worms and inactivate bacteria that had not been ingested, as suggested previously by many authors (Smerda, S.M. et al. (1970), King, C.H. et al. (1991), Ding, G. et al. (1995), Lupi, E. et al. (1995), Anderson, G.L. et al. (2003), Laaberki, M.-H. and Dworkin, J. (2008)). For *E. coli* experiments, chlorination was performed at 10 ppm for 5 minutes, then chlorine residual concentration was measured using the DPD colorimetric method (American Public Health Association (APHA) and American Water Works Association (AWWA) (1998)). Chlorine residual was quenched by adding 0.1 ml of sodium thiosulfate (5% W/V). For *B. subtilis* spore experiments, chlorination was done at 15 ppm for 20 minutes due to the higher resistance of spores to chlorination. Chlorine residual concentration was measured and quenched by adding 0.1 ml of sodium thiosulfate (10% W/V). In both cases, chlorination showed no effect on worm viability as they were observed to maintain motility in the chlorinated suspensions. In addition, free chlorine residuals measured at the end of chlorination treatments indicated minimal chlorine demand from the suspension.

4.2.4.2 Sonication

Ultrasonication was used in order to rupture the cuticle of *C. elegans* and release ingested organisms. For UV inactivation assays, half of the suspension was sonicated before UV exposure and bacterial enumeration, while the other half was sonicated after UV exposure, prior to bacterial enumeration. This strategy allows a better characterization of the protection effect provided by internalization. It allows a comparison of samples that have been through all the same steps (but in a different order), rather than comparing only the inactivation by UV of bacteria located inside nematodes versus inactivation of planktonic bacteria (without nematodes). Sonication on ice was performed in 5-ml subsamples of worm suspension in glass vials using an ultrasonication probe (Cole Parmer, CP 70T) at 15 W for 60 seconds. Sonication time was chosen according to protocols found in literature (King, C.H. et al. (1991), Ding, G. et al. (1995), Caldwell, K.N. et al. (2003), Kenney, S.J. et al. (2004), Kenney, S.J. et al. (2005), Locas, A. et al.

(2007)) and optimized by performing a series of preliminary tests in order to find the minimal sonication time that would allow the breaking up of all worms without inactivating the extracted *E. coli* bacteria. After sonication, a 1-ml sample was filtered (0.45 μm filter, 47 mm, Millipore) and the entire surface of the filter was observed at 200X under the microscope to confirm that all worms had been disrupted (Figure 2d).

4.2.4.3 UV Inactivation Protocol

The suspensions were exposed in open Petri-dishes (9 cm diameter) to UV fluences of 5 and 40 mJ/cm^2 using a collimated beam apparatus equipped with a low-pressure UV lamp emitting at 254 nm (Trojan UV Technologies, London, ON, Canada). The fluence rate was measured using an International Light radiometer (Model IL1400A) coupled to an NIST calibrated sensor for a 254-nm wavelength. The measured collimated beams fluence rate was approximately 0.070 mW/cm^2. The fluence applied in the reactors was calculated as prescribed by the standard method proposed by Bolton, J.R. and Linden, K.G. (2003) and the duration of exposure was adjusted according to the desired fluence. Fluence calculation includes four correction factors which take into consideration: (i) the non-homogeneity of the fluence rate at the surface of the reactors; (ii) the reflection of a fraction of the incident UV light at the air-water interface; (iii) the divergence of the light beams; (iv) the absorbance of the water at 253.7 nm. Absorbance was measured using a spectrophotometer (Cary 100 Scan UV-Visible, Varian, Victoria, Australia) equipped with an integrating sphere (Labsphere, North Sutton, NH). Reactors were placed on stir plates and agitated during irradiation to prevent settling of the worms to the bottom of the Petri dishes.

In order for bacteria/spores to remain as much as possible inside the worms throughout the duration of a complete assay, samples were kept at 4 ^oC between all steps of the protocol to slow down all metabolic processes associated with *C. elegans* digestion (Kenney, S.J. et al. (2005)). Samples were filtered for bacterial enumeration immediately after the last UV exposure and sonication steps to prevent any possible effect of photoreactivation. For the analysis of initial concentration of bacteria, all samples that were not sonicated, i.e. untreated samples ("N_0") and chlorinated samples ("Cl_2") were filtered on a 10 μm isopore membrane filter (# TCTP04700,

Millipore) in order to remove nematodes and therefore prevent defecation of viable bacteria on the culture medium during the 24-h incubation period, which would interfere with the enumeration of external (uningested) bacteria. The 10 μm was shown to effectively retain nematodes without significantly reducing the concentration of planktonic bacteria/spores in the sample. The protocol for UV inactivation assays is summarized in Figure 4.1.

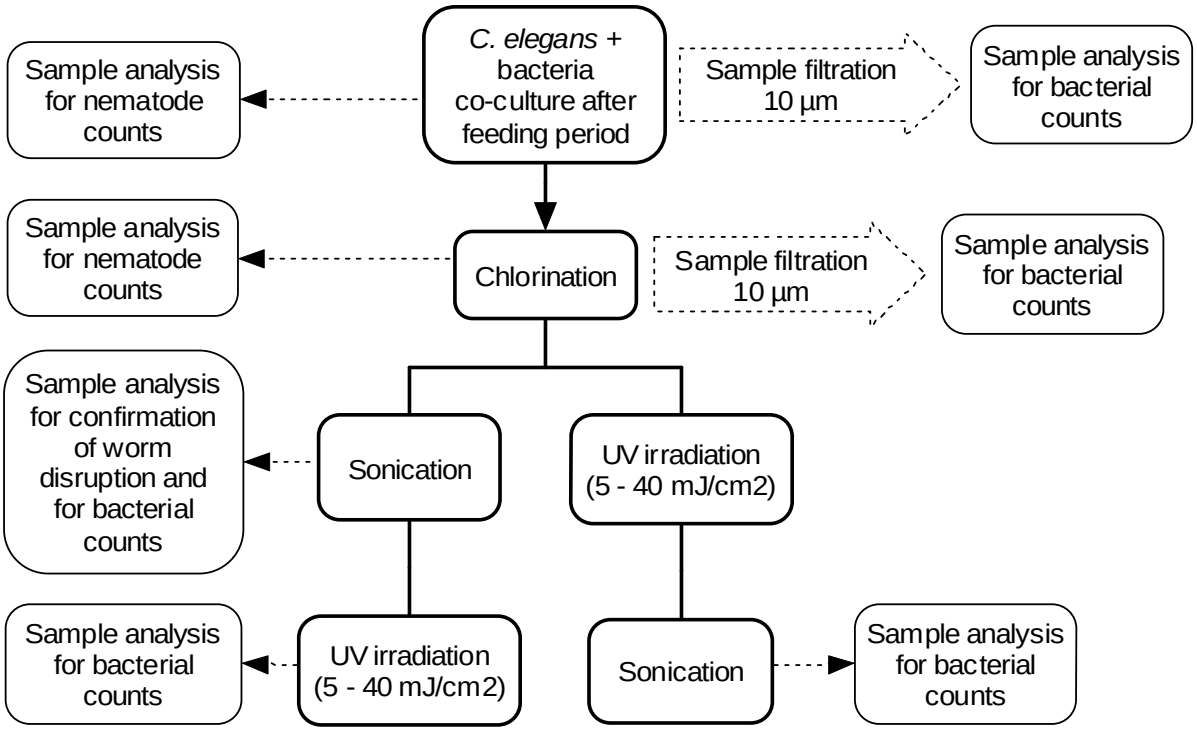

Figure 4.1: Summary of the UV inactivation assays protocol

4.2.4.4 Bacterial Enumeration and Nematode Counts

Nematodes were counted by filtering 1 ml of the suspension on a 0.45 μm filter (Millipore, 47 mm) which was observed under the microscope at 200X. The entire filter surface was observed. In the case of experiments with *E. coli*, the use of bacteria expressing the GFP allowed to visualize internalized *E. coli* inside the gut of *C. elegans* by epifluorescence microscopy (Figure 4.2a, b). As for spores, they were seen as round black holes in the nematode autofluorescent intestinal cells (Figure 4.2c). *E. coli* bacteria were enumerated on duplicate m-Endo agar plates after filtering 1-ml of appropriate dilutions on 0.45 μm filters (Millipore, 47 mm). Petri dishes were incubated at 35 ^{o}C for 24h, based on the standard method for enumeration of total coliforms

((American Public Health Association (APHA) and American Water Works Association (AWWA) (1998)). *B. subtilis* spores were enumerated by filtering appropriate dilutions in duplicates, transferring the filters onto TSB Petri pads, pasteurizing the Petri dishes at 75 ^{o}C for 15 min and incubating at 35 ^{o}C for 24h. The detailed protocol is described in Barbeau, B. et al. (1997).

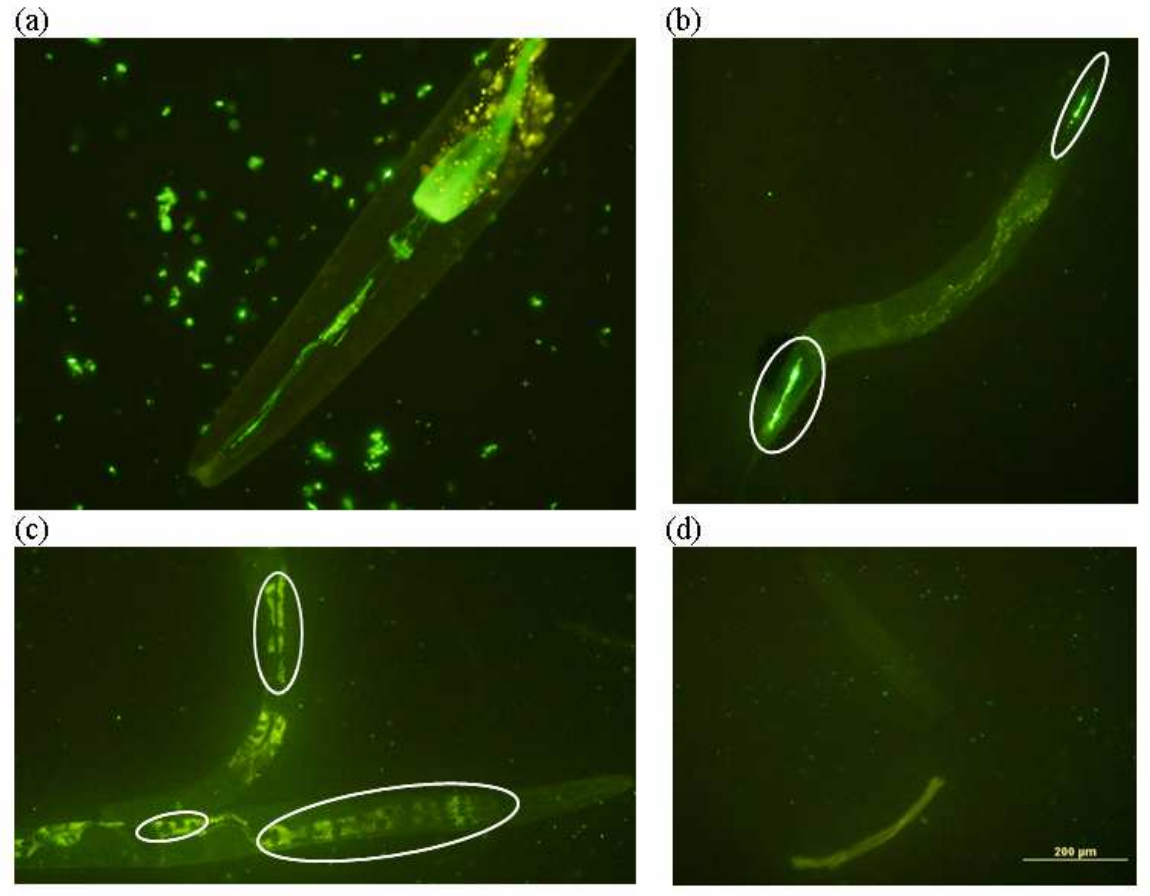

Figure 4.2: (a) *E. coli* OP50 – GFP (green fluorescence) observed inside a *C. elegans* pharynx. Yellow spots are autofluorescence from the nematode's cells. (b) Brightly fluorescing *E. coli* OP50 – GFP bacteria (circled) inside the nematode's pharynx and digestive tract towards the tail. (c) Spores observed as black "holes" (circled) in nematode intestinal cell autofluorescence. (d) All that remains after sonication are bacteria and some nematode debris.

4.3 Results and Discussion

Table 4.1 shows bacterial concentrations measured following the different treatment steps. Disinfection assays were replicated during three successive weeks. Results indicate a very good reproducibility considering that nematode concentrations in the initial suspension varied from one assay to another (see Table 4.2). Yet, the average concentration was found to be 175 nematodes/ml for both *E. coli* (47-357/mL) and *B. subtilis* spore (6-420/mL) assays. Higher nematode concentrations reflect the start of a new generation of worms in the synchronized culture, meaning that both adult worms and young worms (L1 to L2 larval stage worms) were found in the initial suspensions. The variations in nematode concentrations between distinct assays most probably imply variations in both the initial concentration of external bacteria in the co-suspension and in the average number of internalized bacteria per worm (as a consequence of the distribution of worm age in some cultures). However, even though the concentrations of both nematodes and prey differed from one assay to another, the relative protection effect exhibited good reproducibility. The following sections will detail the impact of each treatment step.

4.3.1 Chlorination

Chlorination of the initial co-suspensions was shown to reduce external bacteria/spore concentration by 2.2 logs and 1.6 logs on average for *E. coli* and *B. subtilis* spore experiments, respectively (Table 1, line #2). Therefore, even though the chlorine demands were fairly low (less than 15% of the applied dosage in all cases), not all external bacteria were inactivated by the chlorination treatment, despite the high applied CT in both cases (50 mg·min/L for *E. coli* and 300 mg·min/L for spores). The higher resistance of bacteria and spores to chlorination was not unexpected considering the potential attachment to nematode cuticles.

Tableau 4.1: Measured concentration of (a) *E. coli* bacteria and (b) *B. subtilis* spores at each step of the UV inactivation assay protocol.

	#	Treatment				Measured bacterial concentration			Conclusion (average ± STD)**		Meaning
						Assay 1	Assay 2	Assay 3			
		Cl_2	+ SON	+ UV*	+ SON	Log (cfu/ml)			Log (cfu/ml)		
E. coli	1					5.5	5.4	5.5	(1)	5.5 ± 0.1	Uningested bacteria in initial suspension
	2	**x**				3.3	3.1	3.5	(1) / (2)	2.2 ± 0.2	Inactivation by chlorine
	3	**x**	**x**			5.4	4.6	5.4	(3) – (2)	5.1 ± 0.5	Release of bacteria by sonciation
	4	**x**	**x**	5		3.1	2.0	3.3	(3) / (4)	2.3 ± 0.2	UV inactivation of bacteria extracted from nematodes (in presence of worm debris)
	5	**x**	**x**	40		-0.2	0.3	-0.3	(3) / (5)	5.2 ± 0.8	
	6	**x**		5	**x**	3.0	2.8	5.2	(3) / (6)	1.5 ± 1.1	UV inactivation of bacteria located inside nematodes
	7	**x**		40	**x**	2.1	2.1	3.2	(3) / (7)	2.7 ± 0.6	
***B. subtilis* spores**	1					5.2	4.2	4.3	(1)	4.6 ± 0.6	Uningested bacteria in initial suspension
	2	**x**				3.7	3.0	2.1	(1) / (2)	1.6 ± 0.5	Inactivation by chlorine
	3	**x**	**x**			4.6	4.7	3.4	(3) – (2)	4.2 ± 0.7	Release of bacteria by sonciation
	4	**x**	**x**	5		4.5	4.5	3.3	(3) / (4)	0.1 ± 0.0	UV inactivation of bacteria extracted from nematodes (in presence of worm debris)
	5	**x**	**x**	40		2.2	1.5	1.3	(3) / (5)	2.6 ± 0.5	
	6	**x**		5	**x**	4.6	4.4	3.4	(3) / (6)	0.1 ± 0.2	Inactivation by UV of bacteria located inside nematodes
	7	**x**		40	**x**	3.2	4.2	3.1	(3) / (7)	0.7 ± 0.6	

*Numbers represent the UV fluence applied in mJ/cm^2. **Average calculated from paired data from each assay.

4.3.2 Sonication

Following chlorination, sonication was shown to release on average $10^{5.1}$ and $10^{4.2}$ CFU/ml of *E. coli* bacteria and *B. subtilis* spores, respectively (Table 4.1, line #3). These concentrations are calculated by subtracting the concentration of external bacteria that survived chlorination (Table 4.1, line #2) from the total measured concentration of bacteria after sonication (Table 4.1, line #3). Since the concentration of bacteria remaining in the chlorinated sample was at least 2 orders of magnitude below the concentration measured after sonication, the presence of these external bacteria did not significantly impact our evaluation of the fate of internalized bacteria through the subsequent UV treatment steps.

As can be seen in Table 4.2, nematodes were shown to carry *E. coli* bacteria and *B. subtilis* spores in numbers varying respectively from $10^{2.0} – 10^{3.7}$ and from $10^{1.9} – 10^{2.7}$ CFU/nematode. The initial concentration of *E. coli* or *B. subtilis* spores in the co-cultures was constant among assays and should not have been a restricting factor for nematode feeding. Therefore, we suggest that these variations rather reflect, in the case of co-cultures with *E. coli*, the ability of nematodes to digest the bacteria, which is influenced by the worm's age. In fact, microscopic observation within the framework of this study confirmed previous reports (J.-C. Labbé, personal communication) of the fact that young *C. elegans* digest *E. coli* OP50 easily. This probably explains how a lower average number of bacteria per nematode is found when the nematode concentration is high in the suspension (Assay 2), indicating, as stated earlier, the presence of young worms. As for *B. subtilis* spore assays, the concentration of ingested spores per nematode was generally lower than for *E. coli* co-cultures, which was not surprising considering mainly the much shorter feeding period on spores, and was also less variable, most probably as a consequence of the lesser digestibility of the spores by *C. elegans* (Laaberki, M.-H. and Dworkin, J. 2008). The lower average number of ingested spores per nematode found when the initial suspension contained a higher concentration of worms (Assay 1) might indicate that the smallest worms (L1 and L2 larval stages) do not feed as effectively on spores, which are not recognized as a usual food source for *C. elegans*. These results were compared to other published values in the literature. Laaberki, M.-H. and Dworkin, J. (2008) found an average concentration of about $10^{3.1}$ CFU/nematode when feeding *C. elegans* either with *B. subtilis* spores or *B. anthracis* spores, whereas Kenney, S.J. et al. (2005) found concentrations of $10^{5.2}$ CFU/nematode of *E. coli* OP50.

Meanwhile, Laaberki, M.-H. and Dworkin, J. (2008)), found on average $10^{1.5}$ CFU/worm when feeding *E. coli* OP50 to younger *C. elegans* worms (L4 larval stage). Those numbers can be interpreted as an indication of the capability of nematodes to transport an infectious dose of a human pathogen. In fact, when looking at human infectious doses (ID_{50}) for waterborne pathogens, we find that the ID_{50} are in the order of 10^5 to 10^{10} for most enteropathogenic bacteria (Percival et al. 2004). However, for a highly cytotoxigenic strain of *E. coli* such as strain 0157 H7, the ID_{50} can be as low as 100 cells, whereas less than 30 oocysts of *Cryptosporidium parvum* can create infection in humans (Percival et al. 2004). Therefore, we cannot rule out the possibility, as suggested by {Sifri, Begun, et al. 2005}, that nematodes can potentially act as vehicles for a number of human pathogens through drinking water systems.

Tableau 4.2: Characterization of initial co-suspensions of nematode and bacteria after selected feeding time

		Assay 1	Assay 2	Assay 3	Average
E. coli	Nematode concentration in initial suspension (ind/ml)	120	357	47	175
	Log (bacteria/nematode)	3.3	2.0	3.7	3.0
***B. subtilis* spores**	Nematode concentration in initial suspension (ind/ml)	420	100	6	175
	Log (bacteria/nematode)	1.9	2.7	2.6	2.4

4.3.3 UV Inactivation

In order to assess the impact of the presence of worms and worm debris in the irradiated samples in the performance of UV disinfection, pure planktonic bacteria and spore suspensions (i.e. in the absence of nematodes) were added to phosphate buffer and exposed to various UV fluences. This task yielded two reference curves (Eqs. 1 and 2) describing the UV inactivation kinetics of free *E. coli* and *B. subtilis* spores, respectively.

$$\log\left(\frac{N}{N_o}\right) = -0.4385 \cdot UV_{fluence} \; ; \; R^2 = 0.989 \quad (1)$$

$$\log\left(\frac{N}{N_o}\right) = -0.1163 \cdot UV_{fluence};\ R^2 = 0.997 \quad (2)$$

Both curves are shown on Figure 4.3. Both regressions were statistically significant ($p < 0.001$) with the residuals being normally distributed and the error term homoscedastic according to the Bartlett chi-square test (Statistica, 2008).

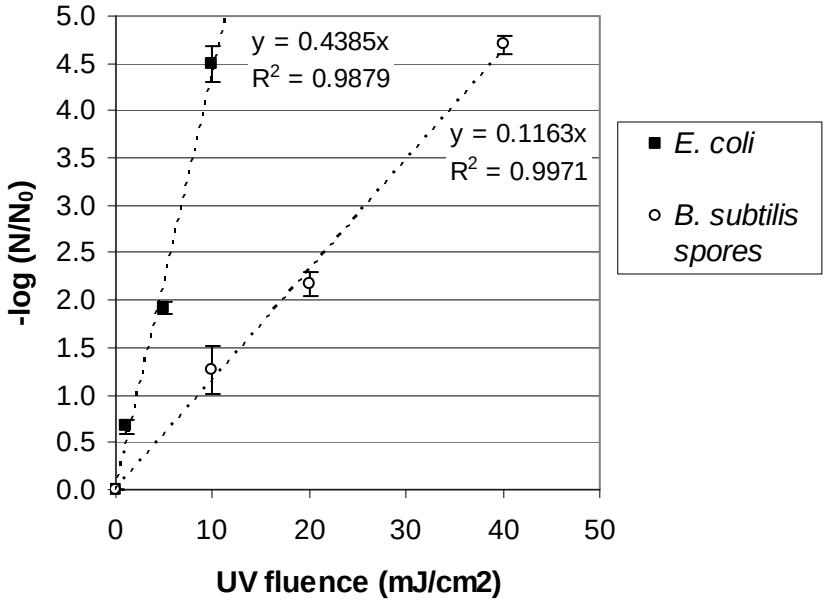

Figure 4.3: Inactivation of *E. coli* OP50 and *B. subtilis* spores ATCC6633 used in this study. Error bars show the standard deviation.

UV inactivation of internalized microorganisms was measured by comparing the final bacteria/spore concentrations in the chlorinated + UV-irradiated + sonicated samples (Table 1, lines #6-7) to the reference chlorinated/sonicated samples (Table 1, line #3). Similarly, UV inactivation of bacteria extracted from nematodes by sonication was calculated by comparing the bacteria concentrations in the chlorinated + sonicated + UV-irradiated samples (Table 1, lines #4-5) to the reference sample (Table 1, line #3). Figure 4.4 compares the UV inactivation results (expressed as log reduction) for these three types of treatments (planktonic bacteria, bacteria + worm debris, internalized bacteria), for both *E. coli* bacteria and *B. subtilis* spores.

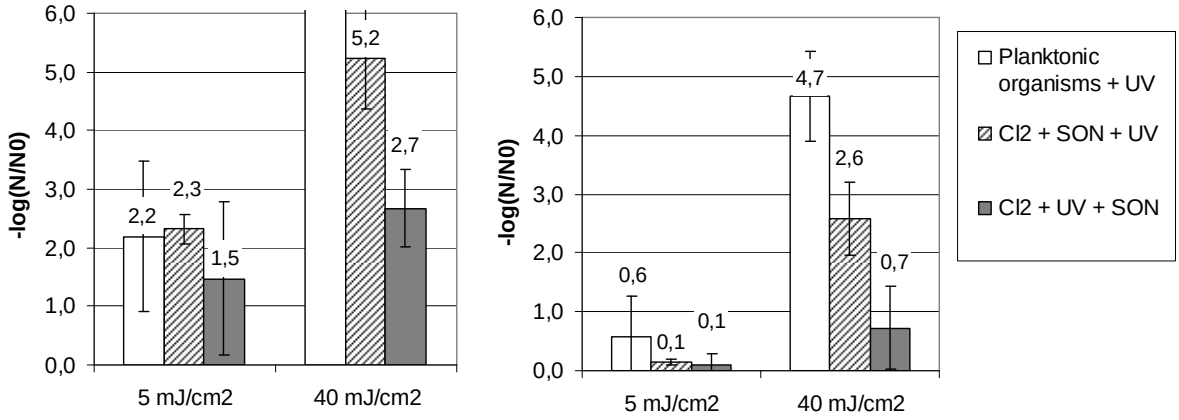

Figure 4.4: UV inactivation (in log) of (a) *E. coli* and (b) *B. subtilis* spores for three treatment conditions: planktonic, chlorinated + sonicated (ruptured) worm suspension ("Cl_2 + SON + UV") or intact worm suspension ("Cl_2 + UV + SON"). Assays were performed at 5 mJ/cm^2 and 40 mJ/cm^2 UV fluences. Error bars indicate the 95% confidence intervals (Eq. 1 & 2 were used for inactivation values of planktonic organisms).

As expected, inactivation data indicate a decrease in UV efficacy against bacteria/spores in both sonicated and intact (chlorinated) worm suspensions as compared to planktonic bacteria. This difference is most notable at the 40 mJ/cm^2, although it was also found to be statistically significant ($p < 0.05$) for *B. subtilis* spores exposed to 5 mJ/cm^2 UV irradiation in the sonicated suspension. For the lower UV dosage in the *E. coli* assays, the experimental error limits our ability to clearly distinguish the targeted phenomenon. For the highest UV dosage of 40 mJ/cm^2, inactivation of *E. coli* should theoretically be virtually complete (> 10 log). In the presence of worm debris, this inactivation was reduced to 5.2 log. However, the internalized *E. coli* were even more resistant with only 2.7 log of inactivation achieved at 40 mJ/cm^2. Similar results were obtained for *B. subtilis* spores. A 4.7 log inactivation of *B. subtilis* spores was achieved at 40 mJ/cm^2 in the absence of nematodes. The presence of worm debris reduced the inactivation to 2.6 log, while internalized spores were even more resistant with only 0.7 log of inactivation.

4.3.4 Evaluation of UV Fluence Reaching Internalized Microorganisms

The concept of biodosimetry was used in order to indirectly quantify the UV fluence reaching the microorganisms located inside the gut of *C. elegans*. The calculation of these UV fluences was based on the measured log inactivation of internalized bacteria/spores (Table 1, lines #6-7), using Equations (1) and (2). The latter, which describes the UV inactivation of planktonic bacteria/spores with respect to the applied UV fluence, can be used inversely to predict the UV fluence corresponding to a measured inactivation. This approach led to the calculation of the internal UV fluences, which were unsurprisingly lower than the dosages applied in the UV experiments, knowing that the efficacy of UV inactivation was lower with internalized bacteria/spores than with planktonic organisms. Finally, in order to obtain the fraction of UV radiation that reached the microorganisms inside the nematode gut, the calculated UV fluences reaching the internalized microorganisms were divided by the applied UV fluences.

Figure 4.5 shows the results of this analysis for *E. coli* bacteria and *B. subtilis* spores at both tested UV fluences. Interestingly, for 3 out of 4 calculated ratios, it was found that 15-18% of the UV fluence applied actually reached the microorganisms located inside nematodes. For one condition (*E. coli* – 5 mJ/cm^2), this ratio was significantly higher (67%). The variability is most pronounced at 5 mJ/cm^2 fluence both for *E. coli* and spores, whereas the range is much narrower at 40 mJ/cm^2. We suggest that the lower inactivation levels obtained at 5 mJ/cm^2 are responsible for the larger uncertainty in the results. In that case, the experimental error arising from the manipulation of a complex biological system involving interaction between two species can becloud the smaller measured inactivation values. However, at a higher UV fluence, higher inactivation levels reduce variability and results indicate that approximately 15 – 16 % of the 40 mJ/cm^2 UV fluence typically applied in water treatment plants do reach organisms that have been ingested by nematodes.

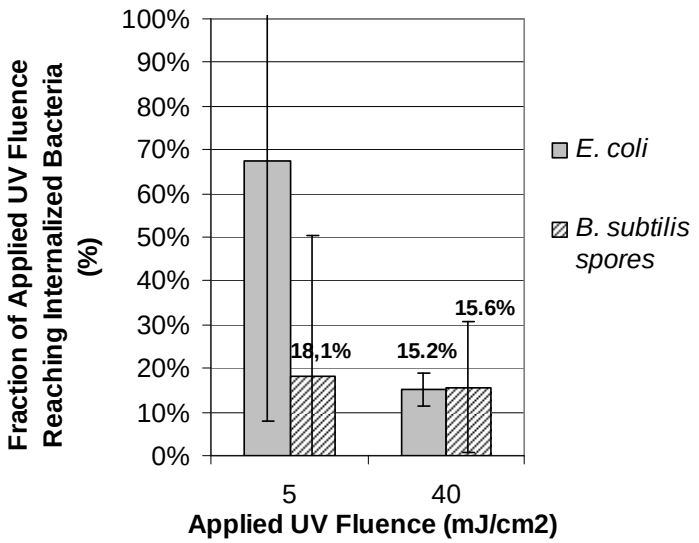

Figure 4.5: Calculated fraction of the applied UV fluence that reaches the bacteria located inside the nematode gut. Error bars show the 95% confidence intervals.

The better reproducibility of spore assays as compared to *E. coli* might be explained by the fact that the bias of bacterial digestion by the nematodes throughout the performing of the assays is significantly reduced with spores, which are unlikely to be digestible by the worms. In that context, spores are a better biodosimeter for measuring the UV fluences inside nematodes. It can also be hypothesised that spore resistance to digestion may be attributed to their thick protein coat layer (Laaberki, M.-H. and Dworkin, J. (2008)). Therefore, spores might act as a better indicator for the fate of similarly robust, thick-walled organisms such as *Cryptosporidium* oocysts when internalized by *C. elegans*. As for the experiments with *E. coli*, it is relevant to point out that the strain used in this study (OP50) is used as a common food source when growing *C. elegans* (Sifri, C.D. et al. (2005), Stiernagle 2006). This information highlights the fact that *E. coli* OP50 is digestible by *C. elegans* under typical conditions. However, as observed in this study, *E. coli* bacteria can accumulate in the gut of adult worms (> 2 days old as reported by Labrousse, A. et al. (2000)) and be less digestible by older worms (> 8 days), at which stage the pharynx starts to be destroyed by the bacteria (Labrousse, A. et al. (2000)). Meanwhile, some human pathogenic strains of *E. coli* have been previously shown to colonize and establish a persistent infection in the intestine of *C. elegans* (Kenney, S.J. et al. (2005)). Such a system, in

which *E. coli* becomes practically unsusceptible to digestion, may be more favourable for evaluating the protection against UV disinfection of enteropathogenic bacteria inside *C. elegans*.

4.3.5 Implication for the Drinking Water Industry

There is an increased awareness in the water industry related to the issue of pathogen internalization by higher organisms. Although such a phenomenon has been described for quite some time (Greub, G. and Raoult, D. (2004)), the role of amoebae in the transmission of Legionnaire's disease has highlighted the genuine microbial risk that can arise from the association of pathogenic bacteria and protozoan. Recently, Loret et al. (2008) have underlined the presence of amoebae and intra-amoebal bacteria through various drinking water treatment plants and found that 100% of raw water samples (n = 25) were positive for amoebae, with concentrations ranging from 1 log MPN/L up to > 3.5 log. 80% of these samples were positive for *Legionella* spp. (detected by PCR). The concentrations of both *Legionella* spp. and amoebae were observed to be significantly lowered by the treatment processes, although a rise was reported after GAC filters. At no point of the treatment chains were those organisms found to be completely eliminated. Both amoebae and *Legionella* spp., including *L. pneumophila*, were detected in water and biofilm samples from distribution systems. The authors stress the point that amoebae might act as "biological by-passes" in drinking water treatment for the bacteria they harbour. Due to the high resistance of amoebal cysts to disinfection, they recommend control strategies based on the physical removal of these organisms. However, granular media filters favour proliferation of higher organisms, including amoebae and nematodes, and their feeding on bacteria. It was suggested long ago that nematodes may be potential vehicles for pathogenic bacteria through drinking water systems (Chang, S.L. et al. (1960), Bertolucci, G.C. et al. (1998)). Nematodes are among the most abundant higher organisms found in granular media filters (Hijnen, W.A.M. et al. (2007)) and concentrations reaching 71 nematodes per litre have been documented in the effluent of GAC filters (Castaldelli, G. et al. (2005)).

Considering that internalization by a nematode offers a nearly absolute protection to chemical disinfection (Ding, G. et al. (1995), {Lupi, Ricci, et al. 1995}, Locas, A. et al. (2007)), it was of

interest to investigate the ability of UV radiation to reach indicator organisms internalized by nematodes, and thus evaluate the potential of UV to serve as an alternative strategy for inactivating internalized pathogens in drinking water. Our findings indicate that it is possible to inactivate internalized organisms with UV radiation, although the effective UV fluences were lowered by a factor of approximately 6 due to absorbance offered by the nematode's body. Nevertheless, this reduction of performance is somewhat modest as compared to the traditional oxidant-based disinfectants, which are essentially ineffective under common water treatment conditions. As UV disinfection is typically used after filtration in the drinking water industry, this process might be an appropriate strategy for addressing this microbiological challenge.

On the other hand, after being irradiated with 40 mJ/cm^2 UV fluence, *C. elegans* could still release 2 – 3 logs of *E. coli* and 3 – 4 logs of *B. subtilis* spores under the experimental conditions in this study. Although this observation suggests a residual microbial risk, the results described herein were obtained while using nematodes with a very high number of internalized bacteria due to laboratory co-culture conditions. It is questionable whether or not such high concentrations could be observed under environmental conditions. The low concentrations of pathogenic microorganisms found in most drinking water sources suggest that pathogen ingestion by higher organisms under natural conditions remains a low probability event. This does not necessarily imply that the defined associated risk can be neglected, since some waterborne pathogens, such as *Campylobacter* or *Cryptosporidium*, can be of concern at low doses, as discussed earlier. Also, some human bacterial pathogens have been shown to be nematode pathogens as well (Sifri, C.D. et al. (2005)) and further shown to colonize the worm intestine after ingestion, therefore establishing the theoretical possibility of finding a high number of bacterial pathogens inside an environmental nematode. It is therefore prudent to consider that a single organism persisting through a water system could potentially harbour a sufficient amount of pathogens to create infection in a human consumer. Other aspects remain poorly understood in the transmission of pathogenic organisms by higher organisms in drinking water, including the possibility of pathogens being naturally released by their hosts in drinking water in a viable and infective state. Smerda et al. (1971) have studied the release of *Salmonella* sp. by natural defecation and reported that, depending on the culture medium used to recover defecated bacteria, the recovery of *Salmonella* varied from 20% to 93.3%. Viable *S. wichita* were freed from nematodes in a tap

water solution by defecation, which reflects drinking water conditions. Kenney et al. (2005) have reported transmission of enteric pathogens by *C. elegans* nematodes to progeny and to a new population of wild-type worms by excretion of viable cells into their environment (at lab-scale). Dennehy et al. (2006) have also reported transmission of bacteriophages between spatially distinct patches of bacteria on agar plate. Therefore, there are indications at laboratory scale that nematodes can transmit both viruses and bacteria to new hosts by natural processes including defecation. Literature reports that bacterivorous nematodes can excrete from 30% to 60% of bacteria ingested in viable form (Chantanao and Jensen 1969). However, it is possible that the excessive concentration of bacteria fed to nematodes in lab-scale experiments might contribute to a higher number of bacteria passing unharmed through the predator's gut due to available food exceeding the digestion capacity of the worms (Chantanao and Jensen 1969). We hypothesize nonetheless that this bias might not be implicated in the case of bacteria creating infections inside nematodes.

Finally, a thorough quantitative microbial risk assessment is still needed to evaluate the importance of this hazard and to determine practical implications in terms of water treatment design, operation and management. While higher organisms are known to be ubiquitous in drinking water systems, studies quantifying internalized pathogen occurrence under field conditions are currently lacking, due to great methodological challenges, leaving a so-called "black box" in the drinking water risk assessment.

4.4 Conclusions

In this study, indicator organisms were used to assess UV radiation's potential for penetrating nematodes by applying the principle of biodosimetry. Our results allow the following conclusions to be reported:

Internalization of *E. coli* bacteria and *B. subtilis* spores by *C. elegans* nematodes was shown to offer significant protection against UV disinfection at the typical fluence of 40 mJ/cm^2 applied in the water treatment industry. This protection was found to represent about 85% of the UV fluence being blocked by the worms, leaving only about 15% of the applied fluence reaching the

internalized organisms. To our knowledge, this is the first report of higher organisms offering protection against UV radiation to internalized microorganisms.

B. subtilis spore results showed less variability than *E. coli*, suggesting that spores might act as a better biodosimeter in such a biological system, being more resistant to digestion by *C. elegans*.

A UV fluence of 40 mJ/cm^2 was sufficient to obtain 2.7 and 0.7 log inactivation of internalized *E. coli* and *B. subtilis* spores. Considering that chemical treatments including chlorination are essentially ineffective in inactivating microorganisms internalized inside nematodes, UV radiation appears to be a superior treatment compared to chemical disinfection in order to address this microbial risk.

Finally, it remains to be investigated whether or not pathogen ingestion and transport by higher organisms at various stages of drinking water systems (including granular filtration and distribution networks) occurs at a significant level. Characterizing the occurrence of this phenomenon will further determine the relevance of integrating this risk when assessing the performance of a drinking water treatment system.

Acknowledgments: The authors acknowledge the Industrial-NSERC Chair in Drinking Water and its industrial partners, namely the City of Montreal, John Meunier Inc. and the City of Laval. Special thanks to Normand Labbé (Polytechnique), Serge Parent (City of Montreal) and Jean-Claude Labbé (University of Montreal) for their support and advice. We also wish to express our gratitude towards Wim Hijnen (KWR Watercycle Research Institute, the Netherlands) for his helpful and meaningful collaboration on this project, in spite of the distance. The nematode and *E. coli* strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

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CHAPITRE 5 PUBLICATION #4 : COMPARAISON OF THE ROLE OF ATTACHMENT, AGGREGATION AND INTERNALIZATION OF MICROORGANISMS IN UVC AND UVA (SOLAR) DISINFECTION

Ce chapitre présente les résultats d'essais expérimentaux visant à mesurer l'impact de deux types de mécanismes de protection des microorganismes face à la désinfection UVA, soit l'agrégation et l'attachement aux particles et l'internalisation par des organismes supérieurs. Dans les deux cas, les travaux réalisés sont basés sur des protocoles préalablement élaborés pour déterminer l'impact de ces mêmes mécanismes de protection face à la désinfection UVC. L'objectif de cette étude vise une comparaison entre l'impact de ces mécanismes de protection dans les deux types de procédés de désinfection UV (UVC et UVA). Cet article a été soumis pour publication à *Water Science and Technology*.

COMPARISON OF THE ROLE OF ATTACHMENT, AGGREGATION AND INTERNALIZATION OF MICROORGANISMS IN UVC AND UVA (SOLAR) DISINFECTION

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Abstract: In this comparative study, the impact of two microbial protective mechanisms against simulated UVA disinfection was assessed by using protocols previously developed for UVC disinfection assays. (i) The impact of natural microorganism aggregation and attachment to particles was assessed by targeting total coliform bacteria in natural surface water samples. (ii)

The impact of bacteria internalization by zooplankton was assessed by using *C. elegans* nematodes as a model host and *E. coli* as a bacterial target for UVA inactivation. Dispersion of natural aggregates by blending prior to UVA exposure was shown to enhance the inactivation rate of total coliforms as compared to untreated raw water, while removal of particles by an 8-µm membrane filtration was not. 24% of the highest applied UVA fluence was found to reach internalized *E. coli* in nematodes. In both parts of this study, only slight differences were found with regard to the impact of protective mechanisms against UVA and UVC bacterial inactivation.

Keywords: Aggregation, *E. coli*, internalization, nematodes, particles, solar disinfection

5.1 Introduction

Solar disinfection is a promising technology which is being increasingly used for providing drinking water in developing communities. SODIS is a simple application of solar disinfection performed in commonly available polyethylene terephtalate (PET) bottles and now recognized by the WHO as an appropriate technology for disinfecting drinking water at the household level (WHO/UNICEF 2005). Moreover, this low-cost process is generally well accepted by developing communities. SODIS relies mostly on the UVA portion of the natural sunlight spectrum. UVC radiation does not reach the surface of the Earth (Diffey 1977) and UVB rays are mostly blocked by the PET plastic bottle (Wegelin et al. (1994), Wegelin et al. (2001)).

Artificial UV irradiation is also widely used in water treatment plants of industrialized countries, mostly for the purpose of disinfection. It is performed by producing UVC radiation at the most germicidal wavelengths of the spectrum (typically 254 nm for low pressure lamps) which alters directly the DNA of the microbial cells in the water. In spite of both processes relying on photolysis, research on solar and UVC disinfection has rarely been paralleled apart from the work of Coohill and Sagripanti 2009.

One potential explanation for this situation lies in the fact that they target very different applications: one is mostly used for treating important flows in municipal water treatment of

industrialized countries, whereas the other is used as a rudimentary means for treating very limited volumes of water in a household setting in the developing world. However, the mechanisms responsible for inactivation are also different. Inactivation arising from UVC photons is thought to originate from DNA dimerization which inhibits reproduction of microorganisms (Mitchell and Nairn (1989)) while UVA disinfection alters microorganisms through the photo-oxidation reactions of nucleic acids via reactive oxygen species (Miller et al. 1999). Apart from bacterial spores, Coohill and Sagripanti (2009) found it difficult to predict UVA sensitivity based on the equivalent UVC information.

A synergistic effect of heat in solar disinfection has been reported when water reaches temperatures above 45-50^{o}C (Wegelin et al. (1994), McGuigan et al. (1998)). However, most solar disinfection research now disregards thermal inactivation. It was reported that for most applications, water temperature does not reach a sufficient level to act synergistically with UVA photolysis (Martin Wegelin, personal communication, 2006). Therefore, under this assumption, UVA disinfection could be viewed as an irradiation process similar to UVC disinfection. In that sense, it becomes interesting to reflect on the various interferences reported for UVC disinfection and try to extend these conclusions to UVA inactivation. This was the basic hypothesis which supported this work.

UV irradiation can be compromised by the presence of particles which may protect microorganisms. Microorganism aggregation is also another mechanism which may lead to a lower efficacy of UV irradiation. Recently, these two issues were studied in drinking water applications where UVC is often applied on unfiltered surface waters. At this time, studies (Kingsford et al. 2005, Caron et al. (2007), Templeton et al. 2010) have observed minimal impacts of turbidity if this value remains below the 5 NTU threshold used in most drinking water guidelines. It also appears that turbidity is not the best indicator of particles interference to UVC inactivation as the inactivation of microorganisms seeded in high turbidity waters (wastewaters or turbidity artificially induced with kaolin) was not strongly impacted (Passantino et al. (2004), Li et al. (2009)) . It is not as much the concentrations of particles than the actual attachment of microorganisms to the later that will dictate the overall impact on UV performance.

In the case of solar disinfection, it has been recommended that SODIS be applied for waters with turbidities lower than 30 NTU (EAWAG/SANDEC, 2008). However, most solar disinfection studies assessing the impact of turbidity were achieved with artificially added turbidity (Joyce et al. (1996), Kehoe et al. (2001)). In these studies, the thermal component of the process ($T^o > 55^oC$) was found to compensate for the loss of UVA transmittance in turbid water samples, therefore allowing high inactivation in waters with artificial turbidity as high as 200 – 300 NTU. McGuigan et al. (1998) suggest that solar disinfection, unlike thermal inactivation, is clearly impacted by high turbidity. Additional assays are warranted to evaluate the impact of natural turbidity on UVA irradiation while excluding the role of pasteurisation on the process performance.

Apart from the impact of particles and aggregation, microorganisms can also find enhanced survival conditions by associating with higher organisms. Many human bacterial pathogens invade amoebae or other protozoa (Barker and Brown (1994), Winiecka-Krusnell and Linder (1999), Winiecka-Krusnell and Linder (2001), Greub and Raoult (2004)), in which they can proliferate and benefit from a protection against harsh environmental conditions. Other higher organisms such as nematodes, rotifers and cladocerans have been reported to feed on a variety of waterborne pathogens in laboratory conditions, which resulted in some cases in the subsequent transport of internalized pathogens and their protection against disinfection, as reviewed by Bichai et al. (2008). In UVC disinfection, internalization by nematodes has been demonstrated to partially protect *E. coli* bacteria and *B. subtilis* spores against irradiation, with a ~85% protection at a typical UV fluence of 40 mJ/cm^2. To our knowledge, the impact of internalization on UVA inactivation has never been studied.

In this study, a comparative evaluation of microbial protection mechanisms on UVA and UVC performance was initiated. Two sets of experiments were conducted in order to assess the impact of two types of microbial protective mechanisms in solar disinfection: (i) aggregation of microorganisms and attachment to particles, and (ii) internalization of microorganisms by higher organisms. This project took advantage of disinfection protocols which were developed in our

group and used to test the impact of turbidity (Caron et al. (2007)) and internalization (Bichai et al. (2009)) on UVC inactivation. Therefore, identical assays were conducted with simulated UVA irradiation in order to test the hypothesis that the protection effects for both types of irradiation were similar.

5.2 Material & Methods

In the first series of assays on turbidity, river water samples were treated more or less extensively to allow distinguishing the impact of particle removal and dispersion of aggregates on the efficacy of UV disinfection. Indigenous total coliforms were selected as the target microorganisms for these assays as they were present at sufficiently high concentrations to allow performing disinfection without the need of spiking the water samples. It is our opinion that this approach provides more representative conditions as the organisms are found in their natural state of aggregation and association to particles.

In the case of microbial internalization, *E. coli* was used as a target for disinfection assays. *Caenorhabditis elegans* nematodes were used as a model host for internalized bacteria. Co-cultures were prepared by feeding nematodes with *E. coli* plated on agar. Co-suspensions were prepared to be exposed to UVA radiation. A sonication protocol was used to disrupt nematodes in order to extract and recover internalized *E. coli* bacteria before or after exposure to UVA irradiation. Both protocols are described in greater details in the following sections.

5.2.1 UVA irradiation and fluence rate measurement

Inactivation assays were conducted using a 15-W UVA lamp with a peak emission at 365 nm (UVP, Upland, CA). Fluence rate was measured with a radiometer (IL1400A, International Light, Newbury, MA) calibrated against a NIST standard with a precision of ±6.5%. The required irradiation time for a given fluence was calculated based on the fluence rate corrected according to the standard method proposed by Bolton and Linden (2003). Absorbance was measured with a spectrophotometer (Cary 100 Scan UV-Visible, Varian, Victoria, Australia) equipped with an

integrating sphere (Labsphere, North Sutton, NH). To allow the irradiation of large volumes of water (50 mL), Petri dishes of 9 cm diameter were used. With this configuration, water depth was 1 cm. Suspensions were exposed to the UVA lamp in gently stirred, open Petri dishes.

5.2.2 Assays on the impact of aggregation and attachment

5.2.2.1 Surface water sampling

Raw water samples were collected from the Mille-Iles River on four occasions during the summer months of 2008. Each sample was characterized for the following physico-chemical parameters: turbidity, pH, alkalinity, hardness, particle counts, TOC and UV absorbance at 254 nm (standard) and 365 nm (UVA lamp emission peak).

5.2.2.2 Dispersion protocol

The protocol used to assess the impact of aggregation of microorganisms and attachment to particles was elaborated by Caron et al. (2007). The protocol allows a comparison between three conditions: (i) non-dispersed microorganisms (ND): raw water samples are submitted to UVA irradiation without any pre-treatment, after which the samples are blended (as described later) to disperse the naturally occurring coliform aggregates prior to enumeration; (ii) dispersed microorganisms (D): samples are blended prior to UVA irradiation to evaluate the role of coliform aggregation and/or embedment on UVA inactivation; and (iii) filtered microorganisms (F8): samples are filtered through 8μm pore membranes, after which the filtered samples are irradiated and then blended to disperse coliforms prior to enumeration. Dispersion is achieved by blending (Blender 7012S, Waring, Torrington, CT) the water for 4 minutes at 8,000 rpm with 100 mg/L Zwittergent 3-12 (Sigma Chemical Co., St-Louis, MO). Two-minute rest intervals follow each minute of blending to minimize any increase in water temperature and the presence of foaming. While the dispersion protocol developed by Caron et al. (2007) was targeting indigenous spores of aerobic spore-forming bacteria with UVC disinfection, a different target was selected in UVA experiments, since indigenous spores did not allow measuring sufficient inactivation levels to compare inactivation kinetics for the purpose of this study (results not

shown). In fact, spores are extremely resistant to solar disinfection (Boyle et al. 2008, Gill and McLoughlin (2007)). Total coliforms were chosen as a common indigenous bacterial indicator occurring in sufficiently high concentrations in surface water and with a similar solar disinfection resistance as for other indicators and pathogens commonly targeted in solar disinfection studies such as *E. coli* and bacteriophages, as well as *Salmonella typhimurium*, *Shigella* and *Enterococci faecalis* (Gill and McLoughlin (2007)).

5.2.2.3 UVA inactivation

The suspensions were exposed to UVA radiation for various exposure durations, leading to UVA doses between 10 and 35 J/cm^2. Such fluences represent exposure to strong solar radiation for durations of approximately < 1 to 3 hours (Ubomba-Jaswa et al. 2009).

5.2.3 Assays on the impact of internalization

The nematode *Caenorhabditis elegans* has been shown to ingest and vector a variety of human pathogens and is commonly used as a biological model to investigate host-pathogen interactions (Bichai et al. (2008)). A protocol was developed to (i) feed nematodes with the target organism *E. coli*, (ii) irradiate the co-suspension with the UVA lamp, and (iii) recover the internalized bateria using sonication for nematode disruption and plate culture for bacterial enumeration. The complete detailed protocol for nematode culture and co-suspension preparation with *E. coli* for inactivation assays is described in Bichai et al. (2009). A strain of *E. coli* expressing the green fluorescent protein (GFP) was used, which allowed to visualize internalized *E. coli* inside the gut of *C. elegans* by epifluorescence microscopy for qualitative control.

5.2.3.1 Preparation of nematode - E. coli co-cultures

A wild type strain (N2) of *C. elegans* was used in this study. Worms were maintained on 5-mm NGM agar plates. GFP-*E. coli* OP50 was cultured at 37^{o}C for 24 h in L-Broth. The NGM agar was surface inoculated with 0.1 ml of a 24-h culture of *E. coli* OP50 – GFP and incubated at 37^{o}C for 24 h to establish confluent growth before transferring the worms. Nematode cultures

were synchronized following the protocol described in details in Bichai et al. (2009) in order to obtain an age-homogenous population of nematodes for performing the inactivation assays. Eggs were then deposited on the surface of an NGM agar plate on which a lawn of *E. coli* OP50 - GFP had formed, and the plates were incubated at room temperature for 3 days to obtain adult worms. Nematodes collected on the third day of incubation were used in all disinfection assays.

5.2.3.2 Preparation for UVA inactivation assays

The experimental protocol described hereafter was replicated on three successive weeks. Each replicate disinfection assay was performed using new synchronized worm cultures. Recovered suspensions were transferred into one sterile 50-ml centrifuge tube and sterile phosphate buffer was added to yield a final volume of 50 ml. The suspension was washed repeatedly by centrifugation and resuspension in sterile phosphate buffer to reduce chlorine demand. The obtained suspension was homogenized by gentle vortexing and then analyzed to measure the initial concentration of bacteria and nematodes. Suspensions were chlorinated using sodium hypochlorite prior to UVA inactivation assays in order to surface-sterilize the worms and inactivate bacteria that had not been ingested, as suggested previously by many authors (Smerda et al. (1970), King et al. (1991), Ding et al. (1995), Lupi et al. (1995), Caldwell et al. (2003), Laaberki and Dworkin 2008). Chlorination was performed at a dosage of 10 mg Cl_2/L for 5 minutes, then free and total chlorine residuals were measured using the DPD colorimetric method (American Public Health Association (APHA) and American Water Works Association (AWWA) (1998)). Chlorine residuals were quenched by adding 0.1 ml of sodium thiosulfate (5% W/V). Chlorination showed no effect on worm viability as they were observed to maintain motility in the chlorinated suspensions. In addition, free chlorine residuals measured at the end of chlorination treatments indicated minimal chlorine demand from the suspension.

5.2.3.3 Nematode disruption protocol

Ultrasonication was used in order to rupture the cuticle of *C. elegans* and release ingested organisms. For UVA inactivation assays, half of the suspension was sonicated before UV exposure and bacterial enumeration, while the other half was sonicated after UV exposure, prior

to bacterial enumeration. This strategy allows a better characterization of the protection effect provided by internalization. It allows (i) a comparison of samples that have been submitted to identical treatments (but in a different order), and (ii) a comparison of the inactivation by UVA of bacteria located inside nematodes versus inactivation of planktonic bacteria (without nematodes). Sonication on ice was performed in 5-ml sub-samples of worm suspension in glass vials using an ultrasonication probe (Cole Parmer, CP 70T) at 15 W for 60 seconds. The optimal sonication protocol was chosen accordingly to Bichai et al. (2009), with the objective to disrupt up all the worms without inactivating the extracted *E. coli* bacteria. After sonication, a 1-ml sample was filtered (0.45 μm filter, 47 mm, Millipore) and the entire surface of the filter was observed at 200X under the microscope to confirm that all worms had been disrupted.

5.2.3.4 UVA inactivation

The suspensions were exposed in open Petri-dishes (9 cm diameter) to UVA fluences of 0.70 and 5.60 J/cm^2. In order to assess the impact of the presence of worms and worm debris in the irradiated samples on the performance of UV disinfection, pure planktonic *E. coli* bacteria suspension (i.e. in the absence of nematodes) was added to phosphate buffer and exposed to the same UVA fluences.

In order for bacteria to remain as much as possible inside the worms throughout the duration of a complete assay, samples were kept at 4^{o}C between all steps of the protocol to slow down all metabolic processes associated with *C. elegans* digestion (Kenney et al. (2005)). Samples were filtered for bacterial enumeration immediately after the last UV exposure and sonication steps to prevent any possible effect of photoreactivation. For the analysis of initial concentration of bacteria, all samples that were not sonicated, i.e. untreated samples and chlorinated samples were filtered on a 10-μm isopore membrane filter (# TCTP04700, Millipore) in order to remove nematodes and therefore prevent defecation of viable bacteria on the culture medium during the 24-h incubation period, which would interfere with the enumeration of external (uningested) bacteria. The 10-μm membrane filter was shown to effectively retain nematodes without significantly reducing the concentration of planktonic bacteria in the sample.

5.2.4 Bacterial Enumeration and Nematode Counts

Nematodes were counted by filtering 1 ml of the suspension on a 0.45 μm filter (Millipore, 47 mm) which was observed under the microscope at 200X. The entire filter surface was observed. *E. coli* and total coliform bacteria were enumerated on duplicate m-Endo agar plates after filtering 1-ml of appropriate dilutions on 0.45 μm filters (Millipore, 47 mm). Petri dishes were incubated at 35^{o}C for 24h, based on the standard method for enumeration of total coliforms ((American Public Health Association (APHA) and American Water Works Association (AWWA) (1998)).

5.3 Results & Discussion

5.3.1 Impact of aggregation and attachment

Physico-chemical quality parameters of the Mille-Iles River water samples are presented in **Table 5.1**. Natural water turbidity (ND samples) varied between 7.6 - 18.9 NTU and particle counts were ~10^5/ml and 10^3 for particles >2.25μm and >10μm, respectively. Such turbidities reflect appropriate water quality for the use of SODIS according to EAWAG/SANDEC (2008). The blending procedure (D) showed little impact on water turbidity and particles, while the filtration (F8) step lowered turbidity to about half of its value (3.4 – 7.9 NTU) as compared to raw water and lowered >2.25μm particle counts by about 2 orders of magnitude, while >10μm were reduced to concentrations of 10^1-10^2 particles/ml. UV absorbance of water samples was found to be much lower at 365 nm than for the wavelength of 254 nm. In fact, UV absorbance at 365 nm was not found to influence significantly the effective UVA dose delivered through the 1-cm deep water samples.

Tableau 5.1: Physico-chemical water quality parameters (Mille-Iles River water, July – August 2008)

	Assay 1			Assay 2			Assay 3			Assay 4		
	ND	**D**	**F8**	**ND**	**D**	**F8**	**ND**	**D**	**F8**	**ND**	**D**	**F8**
Turbidity (NTU)	9.8	9.7	4.4	7.6	7.4	3.4	18.9	20.4	7.9	9.2	11.5	4.4
pH	7.4	7.5	7.6	7.5	7.2	7.9	7.5	-	-	7.5	-	-
Hardness (mg $CaCO_3$/L)	46	-	-	34	-	-	26	-	-	30	-	-
Alcalinity (mg $CaCO_3$/L)	30	-	-	27	-	-	34	-	-	31	-	-
TOC (mg C/L)	7.1	-	-	7.4	-	-	7.5	-	-	8.3	-	-
UV Abs 365nm (cm^{-1})	-	-	-	0.052	-	-	0.071	0.074	0.060	0.062	0.064	0.058
UV Abs 254nm (cm^{-1})	0.223	-	-	0.211	-	-	0.299	-	-	0.289	-	-
Particles >2,25µm (#/ml)	1.06E+05	1.02E+05	4.01E+03	5.51E+04	7.93E+04	3.70E+03	1.78E+05	2.11E+05	3.49E+03	7.84E+04	9.21E+04	1.49E+04
Particules >10µm (#/ml)	2.14E+03	1.27E+03	4.40E+01	9.58E+02	8.39E+02	1.21E+02	1.94E+03	1.90E+03	4.20E+01	1.61E+03	1.11E+03	2.74E+02

Total coliform inactivation by UVA for the 3 treatments applied in the aggregate dispersion / particle removal protocol is presented in **Figure 5.1.** We observed equivalent inactivation kinetics for untreated samples and filtered samples ($p = 0.27$), while dispersed samples were inactivated roughly 1.6 times more efficiently. This result suggests that the protection is mostly attributable to bacterial aggregates rather than particles association since only dispersion favoured a higher inactivation rate. These results partly contrast with the ones of Caron et al. (2007) who studied the UVC inactivation kinetics of indigenous spores in water samples originating from the same river (and sampling location) and treated with the same dispersion protocol. They reported the highest inactivation rate on filtered water, followed by dispersed water with the blending protocol, with the slowest inactivation being found in untreated raw water. A 1.7-fold difference was calculated between the highest (filtered) and lowest (untreated) water samples. Their result is indicative that a fraction of indigenous endospores are not only found as aggregates but also associated to particles. Nevertheless, the overall impact on UV inactivation remains modest in both studies.

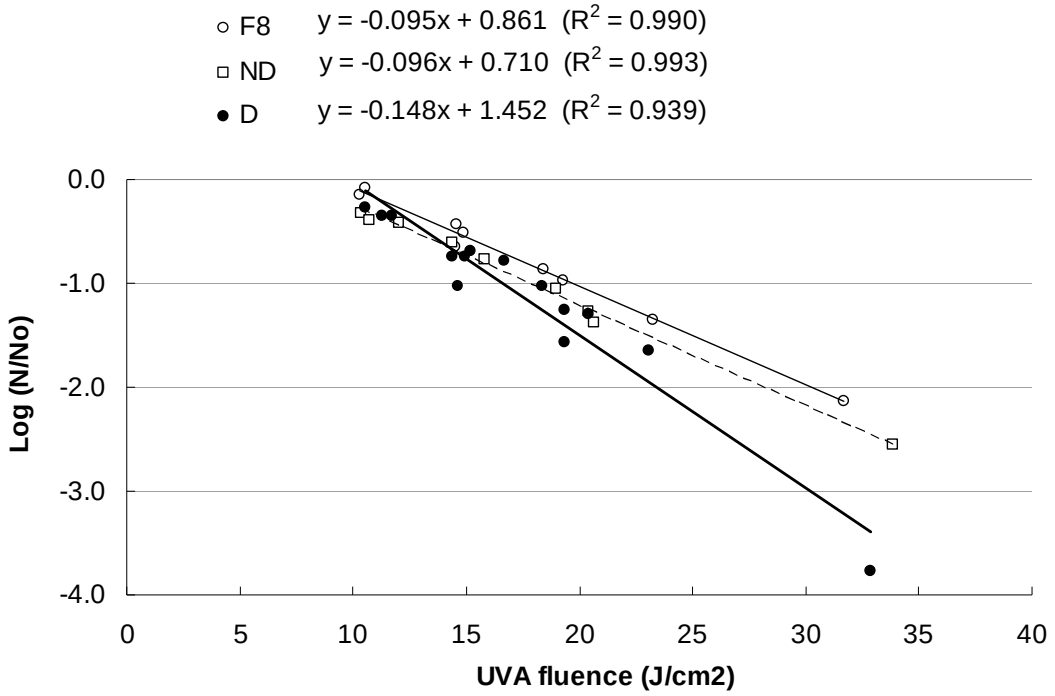

Figure 5.1: UVA (365 nm) inactivation of indigenous total coliforms in non-dispersed (ND), dispersed (D) and filtered (F8) Mille-Iles River waters

5.3.2 Impact of internalization

Results of triplicate internalization experiments with UVA radiation showed as good reproducibility as was obtained for UVC experiments (Bichai et al. (2009)), and are illustrated in **Figure 5.2**.

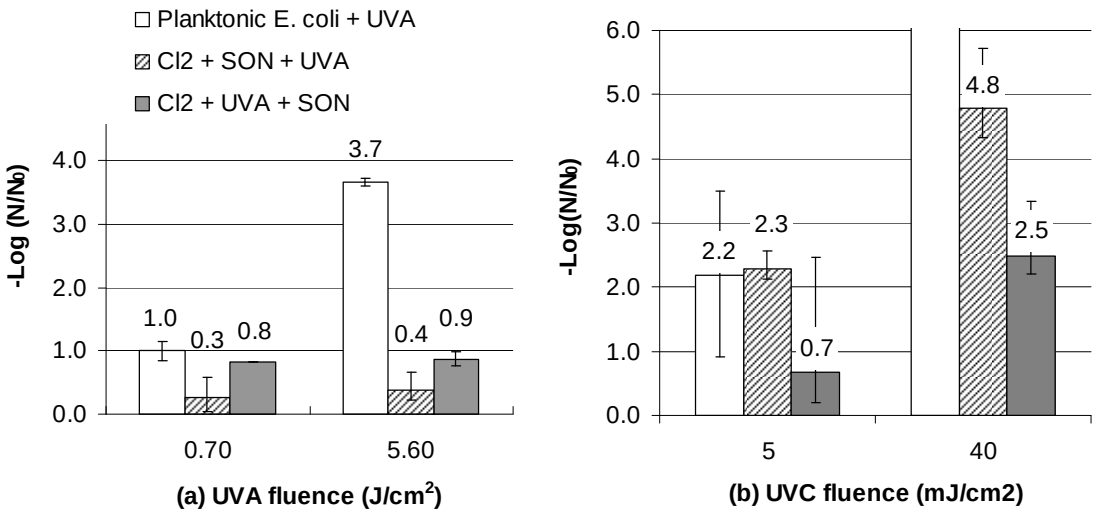

Figure 5.2: (a) UVA and (b) UVC inactivation (in log) of *E. coli* for three treatment conditions: (1) planktonic *E. Coli* cells, (2) chlorinated + sonicated (ruptured) worm suspension (Cl2 + SON + UVA) or (3) intact worm suspension (Cl2 + UVA + SON). Assays were performed at 0.70 and 5.60 mJ/cm^2 UVA fluences (this study) and 5 and 40 mJ/cm2 UVC fluences (Bichai et al. 2009). Error bars indicate 95% confidence intervals.

In both UVA and UVC experiments, the presence of nematodes was shown to interfere with the efficacy of UV inactivation. However, for each of the triplicate assays at both UVA fluences, inactivation was found to be lower when the worm samples were sonicated prior to UVA exposure than when sonication was performed after UVA irradiation of the samples (Fig. 2a). This result was unexpected as it is different than the observations of Bichai et al. (2009) for UVC irradiation (Fig. 2b). We propose that the presence of nematode debris in the suspension exposed

to UVA radiation interfered more with *E. coli* inactivation by UVA than did internalization. UVA photolytic disinfection mechanism has been reported to be influenced by water mineralization and organic matter (Rincón and Pulgarin 2007). It can be hypothesized that the sonication treatment applied to the nematode suspension prior to UVA irradiation might have changed the organic water charcteristics due to the disintegration of the worms in the suspension. Therefore, the disruption of nematodes before UVA may have induced the presence of organic debris favourable to the formation of free radical species, although we did not validate this assumption nor fully characterized the suspensions of disrupted worms.

The estimated fractions of UV irradiation reaching internalized organisms were calculated based on UVA and UVC assays. The UV penetration (in % of applied fluence) reaching the internalized organisms is calculated as the ratio of the log inactivation measured for internalized *E. coli* to the log inactivation of planktonic *E. coli* (reference inactivation level in the absence of nematodes). *E. coli* UVC experiments led to the calculation that ~76% and 16% of the applied fluences of 5 mJ/cm^2 and 40 mJ/cm^2 actually reached internalized cells. For UVA assays, the calculated ratios were similar: 83% for the low fluence of 0.70 J/m^2 and 24% for the higher fluence of 5.60 J/m^2 UVA. For both UVA and UVC experiments, higher ratios (76-83%) were calculated at the lowest than at the highest fluences (16-24%). At the lowest fluence, uncertainty was larger due to the lower inactivation which translates into wider confidence intervals. It could also be argued that the physical protection offered by nematodes is more important for low fluence. In all cases, it may be concluded that internalization by nematodes offers some protection to bacteria against simulated solar (UVA) disinfection.

5.4 Conclusions

In this study, the impact of two microbial protective mechanisms against simulated UVA disinfection was assessed, with the objective to compare the results with past UVC disinfection studies performed using identical protocols.

The impact of natural microorganism aggregation and attachment to particles was assessed by inactivating total coliform bacteria in natural surface water samples (turbidity 7.6 - 18.9 NTU and particle counts ~10^5/ml for particles >2.25μm). Dispersion of natural aggregates by blending prior to UVA exposure was shown to enhance the inactivation rate of total coliforms as compared to untreated raw water, therefore suggesting a similar protection effect due to aggregation of microorganisms in UVA and UVC disinfection processes. Removal of particles by an 8-μm membrane filtration was not shown to enhance UVA inactivation rate of coliforms as opposed to UVC disinfection.

The impact of internalization of bacteria by higher organisms was assessed by using *C. elegans* nematodes as a model host and *E. coli* as a bacterial target for UVA inactivation. Only ~ 24% of an applied UVA fluence of 5.60 J/m^2 actually reached internalized *E. coli* bacteria. We conclude that a similar level of protection is offered to *E. coli* following ingestion by nematodes for both UVA and UVC irradiation.

In both parts of this study, only slight differences were found with regards to the role of protective mechanisms against UVA and UVC bacterial inactivation. While the impacts of physical shielding due to bacterial aggregates or due to internalization inside nematodes were found to be similar for both UVA and UVC disinfection, the influence of particles and nematode debris on UVA and UVC was significantly different. A deeper analysis of the role of water composition in both protocols could further characterize and identify the influence of organic or inorganic compounds involved in the UVA photooxidative inactivation process.

Acknowledgements: The authors acknowledge the work of Jacinthe Mailly for TOC measurements and Mireille Blais for particle counts. The nematode and *E. coli* strains used in this work were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

5.5 References

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CHAPITRE 6 PUBLICATION #5: INTERNALIZATION OF PROTOZOAN (OO)CYSTS BY ZOOPLANKTON IN GRANULAR MEDIA FILTRATION: A QUANTITATIVE MICROBIAL RISK ASSESSMENT IN DRINKING WATER

Ce chapitre présente le développement d'un modèle d'analyse de risque basé sur l'approche QMRA et visant l'évaluation du risque d'infection associé aux microorganismes internalisés dans l'eau potable. Le modèle développé est basé en grande partie sur les travaux présentés au chapitre 3 de cette thèse, décrivant l'occurrence et le transport d'(oo)cystes de protozoaires par le zooplancton dans la filtration granulaire. Cet article a été soumis pour publication dans le *Journal of Water and Health*.

INTERNALIZATION OF PROTOZOAN (OO)CYSTS BY ZOOPLANKTON IN GRANULAR MEDIA FILTRATION : A QUANTITATIVE MICROBIAL RISK ASSESSMENT IN DRINKING WATER

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Abstract: Higher organisms are increasingly perceived as a sanitary concern in drinking water as they are described as potential vehicles and, to some extent, as shields against water disinfection for internalized pathogenic microorganisms. In this study, a quantitative microbial risk

assessment (QMRA) model was developed to evaluate the risk of infection caused by internalized *Cryptosporidium* and *Giardia* (oo)cysts in drinking water. Internalization was described to occur through predation by rotifers in granular filtration. Probability distribution functions were used to describe the variables determining the transport and fate of internalized pathogens. Monte-Carlo simulations were performed using Crystal Ball® to compute the consumers' annual probability of infection under (i) a pilot-scale GAC filtration study conditions, (ii) environmental conditions (lower (oo)cyst load) and (iii) considering the addition of UV disinfection following filtration. When simulating environmental parasite concentrations in water, a mean annual probability of infection of 2.85E-6 and 2.54E-6 was estimated for internalized *Cryptosporidium* and *Giardia* (oo)cysts respectively, lowered to 2.90E-8 and 6.34E-8 when adding UV disinfection. Those results should be viewed as a preliminary effort to better characterize the microbial risk related to internalized pathogens. Refining estimates of some key assumptions used in this model will further help to improve the QMRA.

Keywords: *Cryptosporidium*; granular filtration; *Giardia*; predation; QMRA; zooplankton.

Abbreviations

IP	Internalized pathogens = internalized (oo)cysts
FB	Filter bed of the granular filtration process
DEC	Decimal elimination capacity of the granular filtration process (log)
IR	Ingestion rate (#/rotifer/h)
GAC	Granular activated carbon
Eff	Effluent water of the granular filter
Rot	Rotifers

6.1 Introduction

Higher organisms are ubiquitous in aquatic environments including drinking water systems, and are known as active predators of microorganisms. The significance of predation in drinking water quality depends on the fate of the ingested pathogens: (i) predation is a beneficial process in water treatment when the internalized organisms are digested by the predators; (ii) it may represent a health risk when enteropathogenic microorganisms ingested by zooplankton are persistent and survive internalization so that they are vehicled through water systems and transmitted to consumers; (iii) some human bacterial pathogens are parasitic organisms for protozoan hosts (such as amoebae) inside of which they can multiply and subsequently be transmitted by aerosols. The last category, which includes the famous case of *Legionella*'s regrowth in distribution systems, has received by far the most attention in terms of sanitary concern (Barker & Brown 1994, Winiecka-Krusnell & Linder 1999, Winiecka-Krusnell & Linder 2001, Greub & Raoult 2004, Storey et al. 2004, Loret et al. 2008).

This paper addresses the second category, the internalization of pathogens by zooplankton, their survival to digestion and their subsequent transport through the treatment processes following predation. It emphasizes that apart from amoebae, other zooplankton organisms such as nematodes, rotifers, and cladocerans may also represent a potential sanitary risk in drinking water. Such organisms have been reported to feed on a variety of waterborne pathogens in laboratory conditions, which resulted in some cases in the subsequent transport of internalized pathogens and their protection against disinfection, as reviewed by (Bichai et al. 2008). At pilot-scale, zooplankton was observed to internalize and transport (oo)cysts of *Cryptosporidium* and *Giardia* in GAC filters (Bichai et al. 2010). In a full-scale distribution system, the presence of zooplankton was associated with an unpredicted persistence of total coliforms in chlorinated drinking water (Locas et al. 2007). This phenomenon was explained by the transport of internalized bacteria by nematodes following grazing in sand filters. Understanding the role of higher organisms on the microbial risk is therefore of interest to the drinking water industry.

Quantitative microbial risk assessment (QMRA) is being increasingly used as a tool for decision-making within drinking water quality management frameworks (Haas et al. 1999, Jaidi et al. 2009,

Medema & Smeets 2009). Up to now, apart from a limited number of studies considering the risk associated with amoeba-resisting bacteria (Storey et al. 2004, Loret et al. 2008), the contribution of higher organisms to the persistence and transport (or removal) of pathogens through drinking water treatment processes was not included in QMRA models. In this study, a QMRA model was developed in order to assess the health impact of predation and transport of internalized pathogenic microorganisms by zooplankton in granular media filters, an environment where predation activities are known to be significant. The computational objective of the model is to calculate the consumers' annual probability of infection due to consumption of internalized pathogens in drinking water. In this work, a focus was put on resistant microorganisms, such as *Cryptosporidium* and *Giardia* (oo)cysts, which are reported to be internalized by zooplankton in laboratory conditions (Fayer et al. 2000, Trout et al. 2002, Stott et al. 2003, Huamanchay et al. 2004, Connelly et al. 2007), in granular filtration at pilot-scale (Bichai et al. 2010) and in natural surface waters (Nowosad et al. 2007). Some zooplankton organisms such as rotifers are not yet known to possess enzymes that allow digesting (oo)cysts (Fayer et al. 2000). Zooplankton released from granular filters can therefore transit through treatment processes while protecting internalized (oo)cysts against disinfectants. These internalized (oo)cysts may impose a health risk which can be quantified with a QMRA. It is also of interest to point out that current North American regulations call for the production of treated water containing less than 1 parasite per 100 000 L {United States Environmental Protection Agency (USEPA) 2006 }. In this context, if one internalized (oo)cyst reaches the filtered effluent water, admitting the hypothesis that the probability of surviving subsequent disinfection processes is strong as long as the (oo)cyst remains inside its host, there is a potential for exceeding the quality threshold for safe drinking water provided to consumers even if the process of internalization is rare.

This study presents (i) the rationale and development of the QMRA model for the estimation of the health risk derived from transport of internalized pathogens through granular filtration; (ii) average predicted risks based on Monte Carlo simulations using data on internalization and transport of a pilot-scale GAC filtration study in which high concentrations of *Cryptosporidium* and *Giardia* (oo)cysts were seeded (Bichai et al. 2010); (iii) a sensitivity analysis identifying the most influential input parameters in the model and a discussion on the limits and uncertainties associated with the model; (iv) an extension of the model to predict the risk of internalization

under natural environmental conditions (i.e. low protozoan parasite concentrations), including the possible impact of a subsequent disinfection barrier following filtration: UV disinfection.

6.2 Model description

6.2.1 Model output: Probability of infection by internalized pathogens in treated water, $P_{inf\ (annual)}$

This model aims at calculating the annual probability for an individual ingesting a standard amount of treated drinking water daily to get infected by an internalized *Giardia* or *Cryptsporidium* (oo)cysts. The model was constructed on the basis of the following core hypotheses: (i) internalization of pathogenic microorganisms by higher organisms (predation) occurs in granular filters, and (ii) a fraction of the internalized pathogens (IP) remains inside their host at least until the host is rejected in the filtered effluent water. It is also assumed that internalized organisms released in the filtered effluent (iii) survive post-filtration disinfection using oxidants (ClO_2, Cl_2, O_3 or NH_2Cl) and (iv) will remain as viable and infectious pathogens within their host during treated water transit in the distribution system.

For (oo)cysts of *Cryptosporidium* and *Giardia*, it is generally accepted that the risk of infection can be derived from the exponential dose-response model (Regli et al. 1991). Accordingly to the current USEPA regulation, a targeted annual probability of infection $P_{inf\ (annual)}$ of 10^{-4} or 1 infection per 10,000 people annually must not be exceeded (USEPA 2006). The probability of infection (Haas et al. 1999) is calculated as:

$$P_{\inf\ (annual)} = 1 - (1 - R)^n \qquad (1)$$

where R is the daily risk of infection and the number of exposures n = 365 days.

The daily risk of infection (Eq. 2) can be calculated with the exponential model:

$$R = 1 - \exp(-r \times D) \quad (2)$$

with r is the infectivity parameter describing the host-pathogen interaction, and D is the dose (number) of internalized pathogens ingested daily.

This daily dose, D, is calculated as the product of the concentration of internalized pathogens in the filter effluent (C_{IP_Eff}) times the volume of water ingested daily (V_d) (Eq. 3):

$$D = C_{IP\_Eff} \times V_d \quad (3)$$

When the probability of infection associated to a single exposure (e.g. daily dose) $R << 1$, the annual probability may be expressed as $P_{inf(annual)} = n \times R$ (Haas et al. 1999); therefore in the case of the exponential model, the annual probability of infection can be expressed by considering the cumulative dose as $D \times 365$ (Haas et al. 1999).

$$P_{\inf(annual)} = 1 - (\exp(-r \times C_{IP\_Eff} \times V_d \times 365) \quad (4)$$

Using Crystal Ball® (Decisioneering, USA), Monte-Carlo simulations were performed by sampling randomly 10,000 times in probability distribution functions (PDF) describing the input variables of the model. This number of trials was selected since it resulted in a coefficient of variation of less than 5% for the model output (annual probability of infection).

The daily consumption of drinking water was described using an extreme distribution (see Table 1) with a mean of 1.6 L/d (Barbeau et al. 2000) based on data originating from an epidemiological study in the region of Montreal (Qc, Canada) (Payment et al. 1997). The distribution was

truncated with an absolute minimal value set to 0.18 and a maximum set to 6.25 L/d. The dose-response parameters *r* for *Cryptosporidium* and *Giardia* were described in the model as log-Normal distributions (see Table 6.1) with mean values of 0.096 and 0.017, respectively. These values originate from the USEPA risk assessment used for the development of the LT2ESWTR (USEPA 2002).

The concentration of IP in the effluent water, C_{IP_Eff}, is described in the QMRA model as a forecast distribution. Modeling the effluent concentration of internalized parasites, C_{IP_Eff}, was done by developing a probabilistic equation describing the process of internalization and transport of (oo)cysts by zooplankton in a granular media filter bed. This work will be described in greater details in the following sections.

6.2.2 Conceptual model of predation and transport: predicting the concentration of internalized parasites in the effluent water of granular filters, C_{IP_Eff}

A comprehensive approach of the processes taking place in granular filtration can be based on a mass balance of the pathogens contaminating the filter over a filter run (without backwash). To take into account that a fraction of those pathogens is internalized by zooplankton in the filter bed and escape in the filtered effluent, this mass balance can be expressed over a filter run as:

Pathogen loading (influent) = (Free pathogens + Internalized pathogens) in the filter bed
+ (Free pathogens + Internalized pathogens) in the effluent (5)

Concentrations of internalized pathogens in Eq. (5) are assumed to be much lower than those of free pathogens and at this point in time, there is insufficient data on the fate of free or internalized protozoan (oo)cysts to be able to complete an accurate mass balance on a filter. Modeling the predation mechanisms taking place within the zooplankton population in the filter bed of granular filtration process would require in itself an in-depth study of the grazing activities and the fate of

ingested preys as to determine in which proportion these prey are grazed upon, which proportion is excreted back as free organisms in the filter bed (and thus available for multiple ingestions), and which proportion is digested and therefore removed definitely from the system in a mass balance approach. The proposed model does not intend to describe the complexity of the ecological and microbiological aspects involved in predation, digestion and excretion mechanisms taking place in granular media. Alternatively, a conceptual model was developed based on simplifications of (i) the filter contamination conditions and (ii) the predation, transport and survival of internalized pathogens into filtered waters.

Assumptions were supported with literature data, with most of the quantitative input data derived from a pilot-scale GAC filtration study of (Bichai et al. 2010) describing the internalization of *Cryptosporidium* and *Giardia* (oo)cysts. In that study, 2 parallel GAC filtration columns, in which a mature zooplankton population had developed, were seeded with high concentrations of *Cryptosporidium* and *Giardia* (oo)cysts (1.6×10^5 and 4.8×10^4 (oo)cysts/L, respectively) for 2 hours at the beginning of the study. These conditions can be thought of as the simulation of a brief acute contamination event occurring at the beginning of a filtration cycle. An important raise in internalized pathogen concentrations in the effluent water was observed three weeks after the challenge test was performed. No backwash had been performed during that period. This preliminary observation suggests a retarded breakthrough of internalized (oo)cysts due to the continuous grazing activity of the zooplankton population in the filter bed over time (Bichai et al. 2010). On the basis of that pilot-scale study, it is assumed that the highest concentration of internalized pathogens in the effluent water of a granular media filter would be found at the end of a filtration cycle, right before the backwash.

Simplified conditions of pathogen loading in the filter were simulated for the modeling of the internalization process in the filter. A peak (instantaneous) contamination event was assumed at the beginning of a filter run and the number of pathogen remaining in the filter was calculated based on the pathogen decimal elimination capacity (*DEC*) of the filter. From the time of the contamination event until the end of the filter run, the pathogen concentration immobilized in the filter is assumed more or less constant, and so is the concentration of zooplankton in the filter bed

(predators). The number of internalized pathogens in the filter bed and in the effluent water is assumed to be negligible (nil for the purpose of the model) at the beginning of the filtration cycle, and grazing starts from that point. Based on these assumptions, accumulation of internalized pathogens in zooplankton in the filter bed was described as a linear process based on a simplified constant internalization rate in zooplankton over time. The probabilistic model was developed to predict the highest concentration of internalized pathogens in the effluent water at the end of a filtration cycle (C_{IP_Eff}), which is used as the worst-case scenario for the calculation of the risk of infection in drinking water consumers.

The occurrence of internalized pathogens in filtered water was modeled as a probabilistic chain of events described by 4 variables. (i) The concentration of (oo)cysts (pathogen loading) contaminating the filter bed increases the probability of internalization. (ii) The abundance of predators in the granular media filter bed is also thought to increase this probability. (In this model, predators are assumed to be rotifers, as described later.) Those two first variables describe the ingestion process taking place in the filter bed. However, not all internalized parasites from the filter bed will reach filtered waters: (iii) once ingested, a fraction of the parasites might be either digested (although yet unproven) or excreted; (iv) finally, only a fraction of predators are released in the filtered water, most of them surviving inside the filters. These two last variables describe the transport of internalized pathogens from the filter bed to the filtered effluent. This chain of event was described by Eq. (6):

$$C_{IP\_Eff} = C_{Rot\_FB} \times N_{IP/Rot\_FB} \times S \times F \qquad (6)$$

where C_{Rot_FB} = Concentration of predators (rotifers) in the filter bed

N_{IP/Rot_FB} = Number of internalized pathogens (IP) per rotifer in the FB

S = Probability for an IP to remain and survive inside its host until released in the effluent water

F = Fraction of rotifers from the FB released in the filtered effluent water

These variables are presented and described in greater details in the next sections. Table 6.1 summarizes the probability distribution functions and parameters defining each of the input variables of the model. The development of Eq. (6) is illustrated in Figure 6.1.

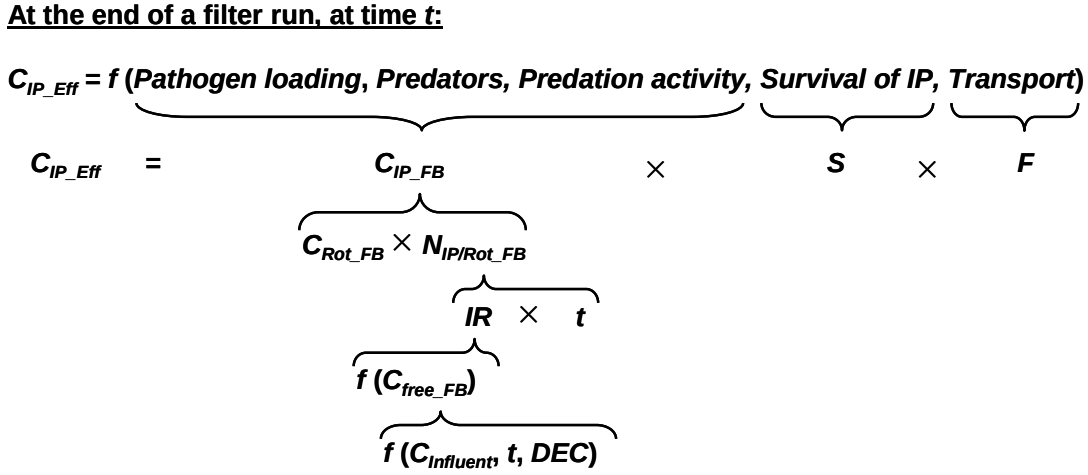

Figure 6.1: Schematic overview of the conceptual model describing the concentration of internalized pathogens (CIP_Eff) in the filtered effluent water

6.2.3 Concentration of rotifers in the filter bed (C_{Rot_FB})

The zooplankton population in the filter bed is assumed to be constant over a filter run and a function of the biomass concentration and the zooplankton load in the raw water. In this study, rotifers are assumed to represent the fraction of higher organisms responsible for most of the ingestion and transport of *Cryptosporidium* and *Giardia* (oo)cysts through granular filtration. In our previous pilot GAC filtration study, rotifers were found to be the dominant population in the filter bed and effluent water and they were hypothesized to be the most probable predators and carriers of (oo)cysts. In previous predation experiments at laboratory scale, *Cryptosporidium* oocysts labelled with fluorescence were seeded in sand samples scraped from the *Schmudzdecke* of slow sand filters of a pilot plant in the Netherlands, and rotifers of the genus *Philodina* and *Rotaria* were the only zooplankton species observed microscopically to contain oocysts after 3h and 24h- predation periods (Y. Dullemont, personal communication). Also, the only report of zooplankton-internalized *Cryptosporidium* (oo)cysts under environmental conditions was for the detection of viable internalized oocysts in rotifers from lake water (Nowosad et al. 2007).

The concentration of rotifers in the filter bed (C_{Rot_FB}) is expressed in terms of number of individuals divided by the volume of interstitial water in the filter bed, considering the porosity of the granular media and the dry density from the GAC pilot study (assumed e = 0.32 and ρ = 0.44 g/ml). Although grazing by zooplankton in the filter bed is thought to occur mostly in the biofilm attached to the granular media, this was considered as part of the interstitial space in the filter bed. The concentration of rotifers in the filter bed is described in the QMRA model by using a triangular distribution with a likeliest value equal to the concentration of rotifers calculated in the GAC filter bed at pilot scale (15.6 rotifers/ml). Very few data in the literature describe zooplankton concentrations inside granular media filters. (Hijnen et al. 2007) reported values of zooplankton concentrations measured at various depths of the filter bed of slow sand filters. As expected, the maximal value, approximately 22 rotifers/ml, was found in the *Schmudzdecke*. The lowest value, 0.4 rotifers/ml, was found in the samples taken at the deepest section of the filter bed. Those data were used to define the upper and lower limits of the triangular distribution describing C_{Rot_FB}, considering that they can represent extreme values that could be found in a granular media filter bed. Since concentrations of rotifers vary on a log-scale, the triangular distribution was used with log-transformed rotifers concentrations in order to define the minimum, mode and maximum values (Table 6.1).

6.2.4 Description of predation activity occurring in the filter bed and calculation of N_{IP/Rot_FB}

The concentration of (oo)cysts available for predation was calculated as the concentration of free (oo)cysts in the filter bed accumulating over one filtration cycle following a peak (instantaneous) contamination event taking place at the beginning of the filtration cycle. A simplification of the breakthrough behaviour of free (oo)cysts in granular filtration was made, assuming that the decimal elimination capacity is attained instantly at time zero and remains constant during the filtration cycle. For example, for a filter that is attributed a 2-log *DEC* for (oo)cysts, the initial concentration of free (oo)cysts in the FB (C_{free_FB}) is considered in the model to be 99% of the total concentration of (oo)cysts that contaminated the FB. The *DEC* values from the loaded GAC

experiments described by Hijnen et al. (2010) were used, with 1.2 and 2.1 log for *Cryptosporidium* and *Giardia*, respectively. As for the concentration of rotifers, free (oo)cysts concentrations in the filter bed are expressed in terms of a number per litre of interstitial water in the filter bed (considering the porosity of the granular media). Therefore, the concentration C_{free_FB} is dependent on the influent concentration ($C_{Influent}$), the duration of the filtration cycle (t), the superficial velocity (v), the porosity (e), the depth of the filter bed (h) and the performance of the filter (DEC) such that:

$$C_{free\_FB} = \frac{C_{Influent} \times t \times v}{h \times e} \times (1 - 10^{-DEC}) \quad (7)$$

We suggest that the probability for internalization of an (oo)cyst by a rotifer in a granular filter bed is increased as the concentration of the prey and/or the predator is higher, raising the odds for both organisms to meet at some point of the filter bed. An internalization rate (IR) was defined in order to characterize the overall accumulation of internalized (oo)cysts inside rotifers in the filter bed.*IR* is influenced by the concentration of predators and prey, the active grazing rate on (oo)cysts by rotifers and their excretion rate. For the needs of the model, *IR* is assumed constant during a given filtration cycle and is computed in relation with the initial concentration of free (oo)cysts in the filter bed (C_{free_FB}) as calculated above (Eq. (7)). The effect of C_{free_FB} on *IR* was included according to our analysis of the data reported by (Stott et al. 2003) in laboratory feeding experiments. Ingestion rates, expressed as *Cryptosporidium* oocysts per hour per predator (the ciliate *Paramecium*), vary as a function of the prey-density concentrations in a log-log manner (see Fig. 2). Based on the data of Stott et al. (2003), IR can be described as a function of the prey concentration (Eq.8):

$$IR = B \times (C_{free\_FB})^{k} \quad (8)$$

Where k and B are empirical parameters, with k = 0.6805 derived from the data of Stott et al. (2003).

The parameter *B* was found to be dependent on the concentration of (oo)cysts. Therefore, the GAC pilot study data was used to estimate the parameters *B* using the concentration of free (oo)cysts in the filter bed calculated from the seeding conditions in the loaded GAC described in Hijnen et al. (2010). As shown in Table 1, the computed values for the parameter *B* are 4.60E-8 and 7.29E-7 for *Cryptosporidium* and *Giardia*, respectively. Based on these values of the coefficient *B*, higher IR are expected for *Giardia* cysts than for *Cryptosporidium* for an identical initial concentration of free (oo)cysts. Since IR is meant in the model to represent the overall rate at which (oo)cysts accumulate inside rotifers, the proposed higher rate for *Giardia* is probably explained by a lower excretion (and possibly digestion) rate, as was reported by Trout et al. (2002). While excretion of *Cryptosporidium* oocysts by rotifers was observed microscopically within 20 minutes (Fayer et al. 2000), no excretion of *Giardia* cysts was observed during the same period (Trout et al. 2002). This observation is also in agreement with the GAC pilot filtration study where higher numbers of internalized *Giardia* cysts than *Cryptosporidium* oocysts were found in the filter bed (0.8 *Cryptosporidium* oocysts and 5.8 *Giardia* cysts per rotifer) 3 weeks after the initial seeding of (oo)cysts (Bichai et al. 2010).

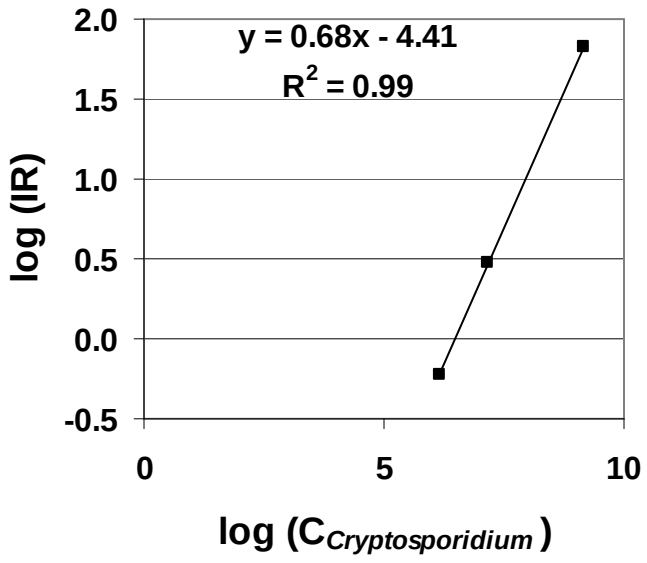

Figure 6.2: Relation between *Cryptosporidium* oocyst concentration and the ingestion rate (*IR*) of *Paramecium* ciliates based on the data by Stott et al. (2003). *IR* is expressed as a number of ingested oocysts per hour per ciliate.

Knowing IR, the average number of internalized (oo)cysts per rotifer in the filter bed at the end of a filtration cycle is calculated as:

$$N_{IP/Rot\_FB} = IR \times t \quad (9)$$

where *IR* is the internalization rate ((oo)cysts per hour per rotifer) and t is the duration of a filtration cycle.

The variable N_{IP/Rot_FB} is described in the model by using a triangular distribution with a likeliest value equal to the number of IP per rotifer calculated in the GAC filter bed at pilot scale: 0.8 and 5.8 (oo)cysts per rotifer for *Cryptosporidium* and *Giardia*, respectively. This variable is assumed to vary on a logarithmic scale. In the absence of additional data, the minimal value for the distribution was set to one order of magnitude below the likeliest value. (i.e. 0.08 and 0.58 (oo)cysts per rotifer). The upper limit of the distribution was set accordingly to laboratory studies in which 6 genera of rotifers were fed with *Cryptoporidium* oocysts (Fayer et al. 2000) and *Giardia* cysts (Trout et al. 2002) in microcosms. In those studies, rotifers were exposed to very high concentrations of (oo)cysts (approx. 10^6/ml or a ~1000-fold higher concentration than in the GAC pilot study). The maximal numbers of (oo)cysts observed microscopically inside rotifers were of 25 and 12 (oo)cysts, for *Cryptosporidium* and *Giardia*, respectively. Those numbers can be considered as the theoretical maximal number of IP that can plausibly be found inside of rotifers.

6.2.5 Transport of rotifers from the filter bed to the effluent water (F)

The variable *F* is meant to describe the transport of rotifers from the filter bed to the effluent water. It represents the fraction (%) of rotifers from the filter bed released into the filtered effluent water and is calculated from the pilot study data as:

$$F = \frac{C_{Rot\_Eff}}{C_{Rot\_FB}} \quad (10)$$

It is assumed that the concentration of rotifers released in filtered water is dependent on the concentration of rotifers in the filter bed in a proportion (*F*) that is independent from the rotifers concentration. *F* is most likely influenced by operating conditions (superficial velocity, filter bed depth, etc.).

There is a wide uncertainty associated with the parameter F, since no data (apart from our pilot study) has been found in the literature to establish a correlation between the concentrations of higher organisms in a granular filter bed and in the filtered effluent. A triangular distribution on a logarithmic scale was used to model the value of *F*. During our pilot study, the value of *F* was calculated as 0.01%, (Bichai et al. 2010) which was used as the likeliest value in the distribution. To define a range of potential *F* values, published data of concentrations of rotifers in filtered waters was used. (Schreiber et al. 1997) reported an average concentration of 1.4 rotifers/L at the effluent of GAC filters from 3 water treatment plants sampled at various durations of filter runs, This average concentration is very close to the concentration found in our GAC pilot filtration study (1.5/L), which suggests that our pilot data is representative of average or likeliest conditions of rotifer concentrations. The rotifers concentrations reported by Schreiber et al. (1997) ranged from <1 to 5488 rotifers/m^3. These two limits were used to define the span of the ratio *F*, with the assumption that the extreme (min/max) rotifer concentrations in the filtered effluent would be associated with the extreme rotifer concentrations in the filter bed (min/max values of the PDF defined earlier, respectively, 0.4 and 22 rotifers/ml). Therefore, the limit values of the PDF for the variable *F* are calculated as a minimum value of 0.00026% and a maximum value of 0.025%.

6.2.6 Probability of persistence and survival of internalized (oo)cysts transported to the effluent water (S)

The variable *S* represents the probability (%) for an IP to remain and survive inside a rotifer until it is released in the effluent water. It can be expressed as:

$$S = \frac{N_{IP/Rot\_Eff}}{N_{IP/Rot\_FB}} \quad (11)$$

The variable N_{IP/Rot_FB} describes the average number of IP per rotifer in the filter bed. The probability *S* is meant to include the issue of digestion and excretion of a portion of IP by rotifers before they reach the effluent water. Those phenomena are not well characterized and the relative importance of digestion and excretion of (oo)cysts by higher organisms is difficult to quantify. In general, digestion is assumed to be most frequent when talking about predation by zooplankton on microorganisms. In biological filtration, predation contributes favourably to the performance of the process, as evidenced by the removal gained through slow sand filtration. However, in the case of protozoan (oo)cysts, rotifers have been shown to excrete *Cryptosporidium* oocysts aggregated in fecal pellets in laboratory conditions (Fayer et al. 2000). Connelly et al. (2007) have also reported ingestion of *C. parvum* oocysts by daphnia, which were observed to excrete oocysts with mostly intact cell wall, although grazing by daphnia was concluded to significantly decrease oocyst infectivity. While the fate of ingested (oo)cysts remains poorly understood, the variable *S* accounts for the experimental evidence (at pilot scale) showing lower IP counts per rotifer in filtered waters as compared to interstitial water in the filter bed.

As for the variable *F*, the only data available to quantify *S* is drawn from our GAC pilot study. *S* is described using a triangular distribution on a logarithmic scale, centered on the value calculated from the GAC pilot filtration data (8.17% for *Cryptosporidium* and 2.43% for *Giardia*). When considering the data from Bichai et al. (2010), concentrations of IP were measured in the effluent water one and 3 weeks after the seeding test and varied within that range of time by 1.1 and 1.5 orders of magnitude for internalized *Cryptosporidium* and *Giardia* (oo)cysts, respectively. It is therefore assumed reasonable that *S* can vary on a log-scale, depending on the duration of the filtration cycle, among other factors which have not been well described up to now. In the absence of data to better characterize variability of *S*, a range of +/- 1 log was arbitrarily set for the value of *S*.

Tableau 6.1: Description of the input variables of the QMRA model based on pilot conditions

Process	Role/Mechanism	Input variables	Units	Description	Assumptions	Distribution
Granular media filtration	Host/Vehicle for IP in the FB	C_{Rot_FB}	#/L	Concentration of rotifers in the FB*	*Rotifers are assumed to be the fraction of the zooplankton population in the FB responsible for internalization and transport of (oo)cysts.*	Triangular= log($C_{Rot\ FB}$) Likeliest= log(1.56E4)[a] Min= log(3.90E2) Max= log(2.22E4)
	Transport of the hosts (rotifers) to the filtered effluent	*F*	%	Fraction of the rotifers from the FB that are released in the filtered effluent	*After predation on (oo)cysts, a fraction of the rotifers from the FB are released into the effluent water.*	Triangular= log(*F*) Likeliest= log(0.01%)[a] Min= log(0.01%) Max= log(0.45%)
	Ingestion of (oo)cysts by rotifers in the FB	C_{free_FB}	#/L	Concentration of free (oo)cysts in the FB at the beginning of the filtration cycle**	*The model simulates a brief peak contamination event happening at the beginning of the filtration cycle. The initial concentration of free (oo)cysts in the FB is calculated considering the total number of (oo)cysts contaminating the FB during a filtration cycle and removing the number of (oo)cysts corresponding to the DEC of the filter.*	Constant (=4.685E6 for *Cryptosporidium*;1.488E6 for *Giardia*)
		IR	#IP/Rotifer/h	Ingestion rate by rotifers in the FB	*Ingestion of (oo)cysts by rotifers is assumed to be at a constant rate through the filtration cycle, which rate is dictated by the initial concentration of (oo)cysts in the filter bed at the beginning of the filtration cycle, right after contamination* (C_{free_FB}). *IR represents the overall rate at which (oo)cysts accumulate inside rotifers through time.*	Constant $IR = B \cdot (C_{free\ FB})^k$ With k = 0.6805 B_{Crypto} = 4.60E-8 [a] $B_{Giardia}$ = 7.29E-7 [a]

		t	h	Duration of a filtration cycle (without backwash)		Constant t = 504 h
		$N_{IP\ Rot\_FB}$	#IP/Rotifer	Number of internalized pathogens (IP) per rotifer in the FB	*The model computes the risk for the highest number of IP per rotifers in the FB. This is assumed to occur at the end of the filtration cycle (worst-case scenario).*	Triangular= $\log(N_{IP\ Rot\_FB})$ Likeliest= $\log(IR \cdot t)$ Min= Likeliest -1 Max_{Crypto} = 25[c] $Max_{Giardia}$ = 12[d]
	Transport of IP to the effluent	S	%	Probability for an IP to remain inside its host until released in the effluent water		Triangular= $\log(S)$ $Likeliest_{Crypto}$= log(8.17%)[a] $Likeliest_{Giardia}$= log(2.43%)[a] Min= Likeliest -1 Max= Likeliest +1
Consumption	Ingestion of IP by human consumer	V_d		Volume of water ingested daily		Max-extreme (likeliest = 1.25; scale = 0.61)[b]
	Infection by IP in human consumer	r_{Crypto}		Dose-response parameter for *Cryptosporidium*		Log-Normal (mean = 0.096; stdev = 1.57E-1)[b]
		$r_{Giardia}$		Dose-response parameter for *Giardia*		Log-Normal (mean = 0.017; stdev = 6.43E-3)[b]

[a]GAC pilot study data by Bichai et al. (2010); [b]Jaidi et al. (2009); [c]Fayer et al. (2000); [d]Trout et al. (2002); [f]USEPA ICR (2002). *Rotifer concentration in the FB is calculated as an average concentration in the interstitial water in the FB (considering the FB porosity). **Concentration of free (oo)cysts in the FB is calculated as an average concentration in the interstitial water in the FB (considering the FB porosity).

6.2.7 Simulating environmental conditions

6.2.7.1 Description of predation activity occurring in the filter bed and calculation of N_{IP/Rot_FB}

In the GAC filtration study performed at pilot-scale by Bichai et al. (2010), very high numbers of *Cryptosporidium* and *Giardia* (oo)cysts (~10^7) were seeded in the filter bed as a challenge test. In the model developed and presented above, the initial concentration of (oo)cysts in the filter bed following this simulated peak contamination is calculated to represent the concentration of (oo)cysts in the interstitial water. This initial concentration (C_{free_FB}) is used to determine the ingestion rate, supposed constant over a filtration cycle, at which rotifers accumulate IP during a filtration cycle.

Under environmental conditions, the initial concentration of (oo)cysts contaminating the filter bed can be expected to be significantly lower than the conditions simulated in the pilot study. A number of assumptions and distributions were defined (see Table 6.2) in order to simulate realistic concentrations of (oo)cysts contaminating the filter bed for a typical drinking water treatment plant:

(1) Log-Normal distributions were used to simulate typical concentrations of *Cryptosporidium* and *Giardia* (oo)cysts in raw water (C_{RW}), with mean values of 0.021 and 0.039 (oo)cysts/L and standard deviations of 12.0 and 20.6 (oo)cysts/L, respectively. These distributions originate from the Information Collection Rule and were used to develop the LT2ESWTR (USEPA 2002). These distributions were truncated at a maximum of 3 standard deviations (99^{th} percentile) to avoid extremely high values. As for the pilot conditions scenario presented above, an instantaneous peak contamination event happening at the beginning of the filtration cycle was simulated in the environmental scenario.

(2) Raw water parasite concentrations are reduced by coagulation/flocculation/sedimentation processes prior to granular filtration. To take into account a more realistic concentration of parasites at the influent of granular filters, removals due to chemically assisted sedimentation (expressed as the variable *CFS*) were simulated with a normal distribution with a mean of 1.9 log

and standard deviation of 0.9 log for *Cryptosporidium*, and a mean of 1.6 log and standard deviation of 0.9 log for *Giardia* (Hijnen and Medema, 2007). Those distributions were truncated at 0.4 and 3.8 log for *Cryptosporidium* and 0.3 and 2.9 log for *Giardia* based on the ranges of published values surveyed by Hjinen and Medema (2007).

(3) The initial concentration of free (oo)cysts in the FB (C_{free_FB}) is calculated as the fraction of the influent (oo)cysts retained in the filter bed during one filtration cycle. This number was divided by the total volume of interstitial water to yield a concentration. For these simulations, typical decimal removals (*DEC*) for granular media filtration were simulated using two normal distributions, which were characterized based on the review by Hijnen and Medema (2007). Mean values of 1.8 and 1.7 log were used for *Cryptosporidium* and *Giardia*, respectively, with standard deviations of 1.3 and 1.1 log. These distributions were limited to minimal and maximal values of 0.0 – 5.5 and 0.0 – 4.1 for *Cryptosporidium* and *Giardia*, respectively (Table 6.2).

(4) The following typical granular filtration operating conditions were set for the simulation: (i) a filtration velocity (v) of 7.5 m/h, (ii) a filter bed depth (h) of 1 m and (iii) a filtration cycle duration (t) of 72 h. A porosity (e) of 32% (same as the GAC pilot-scale filter) was used in order to allow an easier comparison basis with the risk assessment performed in the pilot-scale conditions. A high peak contamination event happening at the beginning of the filtration cycle was simulated, corresponding to the total number of (oo)cysts that would have contaminated the filter bed during a filtration cycle at typical raw water concentrations. The initial concentration of (oo)cysts in the filter bed (C_{free_FB}) used for the ingestion rate computation is calculated according to Equation (12) and is expressed as a number of (oo)cysts by litre of interstitial water in the filter bed.

$$C_{free\_FB} = C_{RW} \times 10^{-CFS} \times \frac{t \times v}{h \times e} \times (1 - 10^{-DEC}) \qquad (12)$$

The calculation of the (oo)cyst ingestion rate (*IR*) by rotifers is based on this initial concentration C_{free_FB} using Equation (8). The number of IP per rotifer in the filter bed at the end of the filtration cycle (N_{IP/Rot_FB}) was described in the model by using a triangular distribution on a logarithmic scale, as stated earlier. In the environmental simulations, the likeliest value of the triangular

distribution is calculated on the basis on the ingestion rate computed as described above and using Equation (9). In the absence of additional data, the minimal value for the distribution was set to one order of magnitude (1 log) below the likeliest value, following the same reasoning as in the pilot model construction. To reflect the fact that the number of IP per rotifer in the filter bed is shifted to a lower range in environmental conditions, the maximal value of the PDF defined in the pilot scenario was also shifted to a lower value for environmental simulations, by maintaining constant the ratio of the maximal value to the likeliest value (25/0.8 and 12/5.8 for *Cryptosporidium* and *Giardia*, respectively). Therefore, maximal values of the PDF describing N_{IP/Rot_FB} were calculated by applying a factor of 31 and 2.1 to the mode of *Cryptosporidium* and *Giardia* distributions, respectively. The distribution was truncated at an absolute maximal value of N_{IP/Rot_FB} equal to the ratio of the initial concentration of (oo)cysts in the filter bed to the concentration of rotifers in the filter bed (C_{free_FB}/ C_{Rot_FB}), in order to reflect the impossibility (zero probability) of having more internalized (oo)cysts than the total number of (oo)cysts contaminating the filter bed.

6.2.7.2 Inactivation by a post-filtration disinfection barrier: UV inactivation

Simulations under environmental conditions were also performed while adding a final UV disinfection step. Unlike chemical disinfection treatments, UV disinfection has been demonstrated to have a potential for inactivating zooplankton-internalized microorganisms (Bichai et al. 2009). A constant fluence of 40 mJ/cm^2 was chosen as a representative operating condition in the water industry. Bichai et al. (2009) showed that approximately 15.6 ± 7.5 % of a 40 mJ/cm^2 applied UV fluence was able to reach *B. subtilis* spores internalized by nematodes. These parameters were used to define a normal distribution (*UV_reach*) describing the fraction of UV irradiation reaching the internalized organisms. The distribution had to be truncated to an absolute minimum value of 6.4% for *Cryptosporidium* and to 2.5% for *Giardia* in order to avoid having negative log inactivation values by UV in the model.

The log inactivation (*UV_inact*) achieved by the effective UV fluence was calculated accordingly to Eq. (13) and (14) ($R^2 > 0.99$) which were derived using the data provided by the USEPA (2006).

$$UV\_inact_{(Cryptosporidium)} = 1.3422 \times \ln(UV\_fluence) - 0.2547 \quad (13)$$

$$UV\_inact_{(Giardia)} = 1.2706 \times \ln(UV\_fluence) + 0.0053 \quad (14)$$

$$\text{where} \quad UV\_fluence = UV\_reach \times 40 \text{ mJ/cm}^2 \quad (15)$$

The concentration of IP in the effluent water of granular media filtration is therefore lowered by the UV disinfection process in such way that the concentration of IP in the treated water (C_{IP_DW}) can be estimated as:

$$C_{IP\_DW} = C_{IP\_Eff} \times 10^{-UV\_inact} \quad (16)$$

Computations of the annual probability of infection associated to internalized pathogens in environmental conditions were performed with Equation (4), using either (i) the concentration of IP in filtered water without subsequent UV disinfection (C_{IP_Eff}) or (ii) the modified concentration of IP in drinking water (C_{IP_DW}) calculated with Equation (15) in order to evaluate the potential of a typical UV disinfection barrier (40 mJ/cm^2) for reducing the risk associated to internalized pathogens in drinking water.

All other input variables implicated in the calculation of P_{inf_annual} under environmental conditions are the same as the ones presented for the pilot scenario (see Table 6.1).

Tableau 6.2: Description of the input variables of the QMRA model for simulations under environmental conditions

Process	Role/Mechanism	Input variables	Units	Description	Distribution / Calculation
Granular filtration	Ingestion of (oo)cysts by rotifers in the FB	C_{RW}	#/L	Concentration of (oo)cysts in raw water	Log-Normal *Cryptosporidium*: Mean = 0.02065; Stdev = 11.977 *Giardia*: Mean = 0.03916; Stdev 20.635 [f]
		CFS	log	Removal of (oo)cysts prior to filtration (Coagulation-Floculation-Sedimentation)	Normal *Cryptosporidium*: Mean = 1.9; Stdev = 0.9 [g] *Giardia*: Mean = 1.6; Stdev = 0.9 [g]
		DEC	log	Decimal elimination capacity of the granular filtration process	Normal *Cryptosporidium*: Mean = 1.8; Stdev = 1.3 [g] *Giardia*: Mean = 1.7; Stdev = 1.1 [g]
		C_{free_FB}	#/L	Concentration of free (oo)cysts in the FB at the beginning of the filtration cycle**	$C_{free\_FB} = C_{RW} * (10^{-CFS}) * (t * v) / (h * e) * (1 - 10^{-DEC})$
		IR	#IP/Rotifer/h	Ingestion rate by rotifers in the FB	$IR = B \cdot (C_{free\_FB})^k$ With k = 0.6805 B_{Crypto} = 4.60E-8 [a] $B_{Giardia}$ = 7.29E-7 [a]
		t	h	Duration of a filtration cycle (without backwash)	t = 72 h

		N_{IP/Rot_FB}	#IP/Rotifer	Number of internalized pathogens (IP) per rotifer in the FB	Triangular= $\log(N_{IP/Rot\_FB})$ Likeliest= $\log(IR \cdot t)$ Min= Likeliest -1 Max_{Crypto} = 31.133*Likeliest[ac] $Max_{Giardia}$ = 2.0586*Likeliest[ad]
Disinfection	Inactivation of IP by UV disinfection	*UV_reach*	%	Percentage of UV fluence reaching IP in rotifers	Normal (Mean = 15.6% ; Stdev = 7.5%)[e]
		UV_fluence	mJ/cm2	UV fluence effective for inactivation internalized (oo)cysts	UV_fluence = UV_reach * 40
		UV_inact	log	Inactivation of free (not internalized) (oo)cysts	*Cryptosporidium*: UV_inact = 1.3422·ln(UV_fluence) - 0.2547[h] *Giardia*: UV_inact = 1.2706·ln(UV_fluence) + 0.0053[h]
		C_{IP_DW}	#IP/L	Concentration of IP in drinking water treated with UV	$C_{IP\_DW} = C_{IP\_Eff} * 10^{(-UV\_inact)}$

[a]GAC pilot study data by Bichai et al. (2010); [c]Fayer et al. (2000); [d]Trout et al. (2002); [e]Bichai et al. (2009); [f]USEPA ICR (2002); [g]Hijnen and Medema (2007); [h]USEPA UV guidance manual (2006); **Concentration of free (oo)cysts in the FB is calculated as an average concentration in the interstitial water in the FB (considering the FB porosity).

6.3 Results and discussion

6.3.1 Point estimate risk calculations derived from the pilot-scale filtration scenario

Point estimates of the annual probability of infection by internalized (oo)cysts of *Cryptosporidium* and *Giardia*, based on data from the pilot-scale GAC filtration study by Bichai et al. (2010), are presented in Table 6.3.

Tableau 6.3: Point estimate risk calculations based on pilot-scale filtration data

	Cryptosporidium	*Giardia*
C_{IP_Eff} (#/L)	0.100	0.208
r	0.096	0.017
V_d (L)	1.6	1.6
$P_{inf\ (annual)}$	9.96E-1	8.73E-1

The annual probability of infection was found to be very close to 100%. In the pilot filtration study, very high concentrations of (oo)cysts were seeded in the filter bed and the concentration of internalized pathogens were measured after a long operation time of the filters without backwash (3 weeks). These conditions represented a worst-case scenario as the filtration cycle was long, the seeded concentration was high and there were no subsequent disinfection barriers following filtration. The concentration of internalized (oo)cysts in the effluent water is in fact about one order of magnitude higher than the typical concentrations of free (oo)cysts in raw water (USEPA 2002), and therefore roughly 4 orders of magnitude higher than expected concentrations of free (oo)cysts in filtered effluent water after conventional treatment. Therefore, a very high probability of infection is expected to be computed by the model at such high IP concentrations in filtered water.

6.3.2 Monte-Carlo simulations based on pilot-scale filtration data and environmental conditions

Results of the simulations performed with the model using pilot-scale filtration data and environmental data are presented in Figure 3.

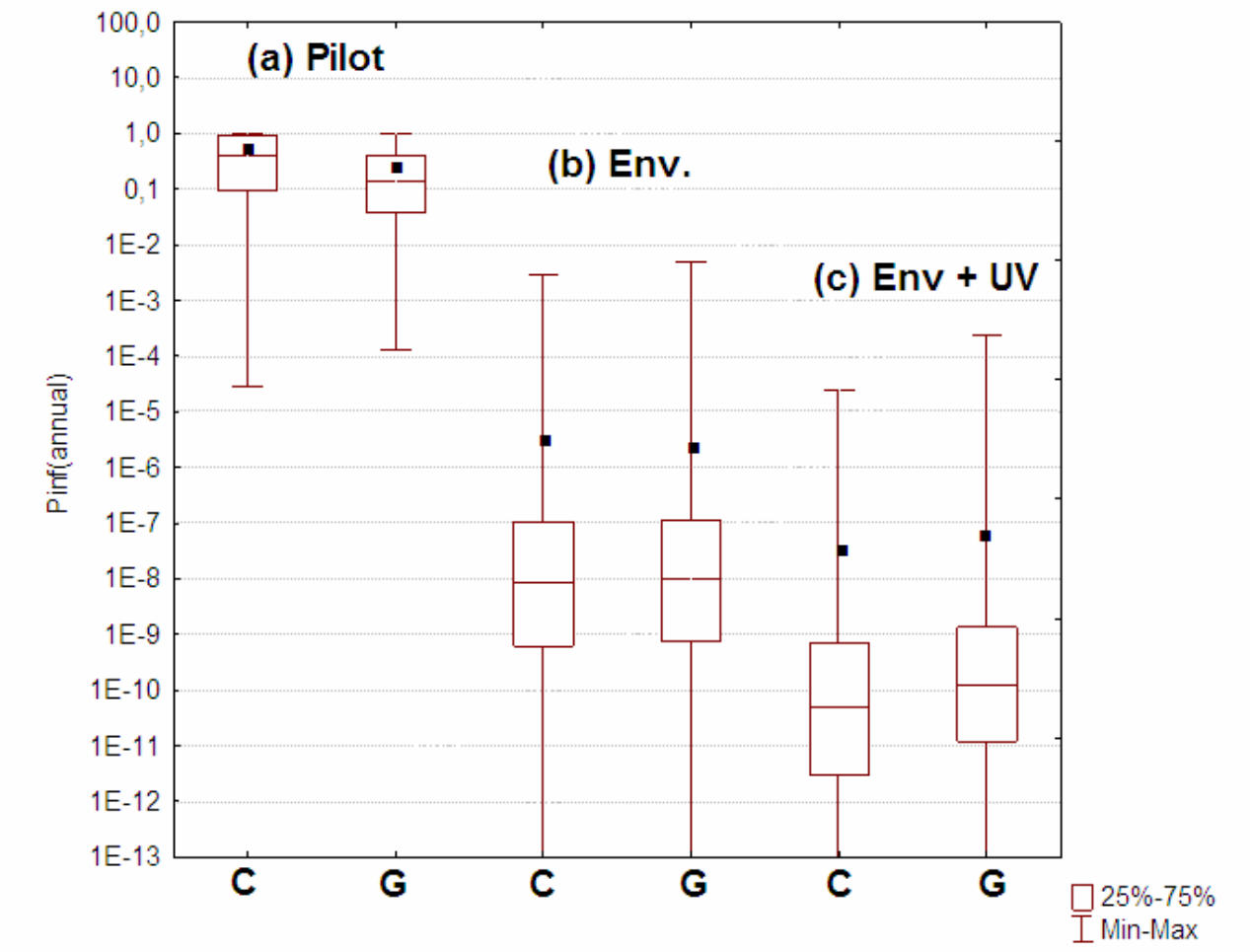

Figure 6.3: Whisker boxes show the 0, 25, 50, 75 and 100th percentiles and the mean value (■) of the annual probability of infection associated to internalized *Cryptosporidium* and *Giardia* (oo)cysts in (a) GAC pilot-scale filtration conditions, (b) environmental conditions, and (c) environmental conditions with the addition of a 40 mJ/cm^2 UV disinfection following granular filtration.

Figure 3 reveals that the uncertainty of the estimated risk values is high, especially under environmental conditions. This is demonstrated by a wide range in each scenario but also by the large difference between mean values and the 50th percentile. Such high uncertainty can be explained by studying the contribution of each variable to the computed risk, as will be presented later in the sensitivity analysis.

Tableau 6.4: Mean annual probability *(with 95% confidence interval)* of infection associated to internalized (oo)cysts in drinking water

Internalized pathogen	Pilot conditions	Environmental conditions	Environmental conditions + UV disinfection
Cryptosporidium	4.76E-01 *(9.49E-03; 1.00E00)*	2.85E-06 *(1.23E-11; 3.61E-06)*	2.90E-08 *(8.10E-14; 2.89E-08)*
Giardia	2.64E-01 *(6.02E-03; 9.36E-01)*	2.54E-06 *(1.96E-11; 3.49E-06)*	6.34E-08 *(3.65E-13; 4.65E-08)*

Table 6.4 shows the mean annual probability of infection associated to internalized (oo)cysts in drinking water under pilot-scale and environmental conditions as computed by the model developed in this study. The pilot-data based simulations reveal a very high risk of infection associated to both internalized *Cryptosporidium* and *Giardia* (oo)cysts, similar to the point estimate risks calculated above. In those simulations, the mean value of the variable N_{IP/Rot_FB} was 2.31 and 4.18 internalized (oo)cysts per rotifer in the filter bed, for *Cryptosporidium* and *Giardia* simulations, respectively. In simulations under environmental conditions, the mean annual probability of infection associated to internalized (oo)cysts is found to be lower than the USEPA standard threshold of 10^{-4} infection/year, both for *Cryptosporidium* and *Giardia*, even without the use of UV disinfection. The addition of a UV disinfection barrier operated at a 40 mJ/cm^2 fluence was shown to reduce the mean risk of infection by internalized (oo)cysts by 2.0 log for *Cryptosporidium* and by 1.6 log for *Giardia*. In environmental simulations, mean values for N_{IP/Rot_FB} were found to be in the order of 10^{-6} and 10^{-5} internalized *Cryptosporidium* and *Giardia*

(oo)cysts per rotifer in the filter bed, respectively. Of course, as the accumulation of IP in rotifers is assumed to be linear through time, the 3-day filtration cycle is expected to result in lower average counts of IP per rotifer at the end of the filtration cycle, as compared to the 3-week filtration cycle of the pilot study GAC filters. However, those environmental numbers also reflect the much lower grazing rates within the rotifer population exposed to environmental concentrations of *Cryptosporidium* and *Giardia* (oo)cysts contaminating the filter bed. These concentrations were estimated by the model to be in average, respectively, ~10^7 and 10^6 lower in simulated environmental conditions vs pilot-scale conditions. Therefore, under the environmental conditions simulated by the model, less than one rotifer out of 10^6 would be expected to contain an internalized *Cryptosporidium* (oo)cyst in the filter bed at the end of the filtration cycle. Considering the average rotifer concentrations generated by the model (around 7200 -7300 rotifers/L in the interstitial water), this would represent ~3-4 internalized *Cryptosporidium* oocysts and ~40-50 internalized *Giardia* cysts per cubic meter of granular media in the filter, considering a 32% porosity. Those numbers of internalized (oo)cysts can be compared to the average free (oo)cyst concentrations estimated by the model to be around 100-200 and 700-1000 *Cryptosporidium* and *Giardia* (oo)cysts per cubic meter of granular media in the filter bed, respectively.

An accurate characterization of the remobilisation of internalized (oo)cysts vehicled by rotifers in granular media filtration, as compared to the breakthrough behaviour of free (oo)cysts, could help defining the relative risk associated to internalized pathogens. The remobilisation of internalized (oo)cysts and their transmission to the effluent water was described by the variables *F* (release of rotifers in filtered effluent) and *S* (survival of internalized (oo)cysts within a rotifer). These variables were computed by the model as similar average values under both pilot-scale and environmental conditions since they were defined as being independent from the initial free (oo)cyst concentration contaminating the filter bed. For the variable *F*, a mean value of 0.01% was computed as the fraction of the total predators leaving the filter bed. For the variable *S*, mean values of 12.4% and 3.6% resulted from the simulations, representing, respectively, the percentages of the (oo)cysts of *Cryptosporidium* and *Giardia* internalized in the filter bed that would still be found inside the rotifers released in the effluent water. Those values suggest that only one *Cryptosporidium* oocyst out of 810,000 internalized in the filter bed (1 out of 270,000 for *Giardia* cysts) would actually end up in the filtered effluent.

6.3.3 Use of the model for environmental conditions: uncertainties and discussion

In the elaboration of this QMRA, many assumptions had to be made when characterizing the variables implicated in the computation of the annual probability of infection associated with internalized (oo)cysts of *Cryptosporidium* and *Giardia* in drinking water. A sensitivity analysis was conducted in order to portray the importance of each variable in the risk computation.

For the pilot-scale scenario, all input variables are important in characterizing the risks, exception made of the daily volume of water ingested and the infectivity parameter for *Giardia* (see Table 6.5) which range is narrower than for *Cryptosporidium* as the dose-response was developed based only on one strain of *Giardia* as opposed to three strains for *Cryptosporidium*. Uncertainties will be further discussed for simulations performed under environmental conditions in this section.

Tableau 6.5: Sensitivity analysis for Monte Carlo simulations performed under (a) pilot-scale seeding test conditions; (b) environmental parasite concentration conditions; (c) environmental conditions with the addition of a 40 mJ/cm^2 UV disinfection following granular filtration

PILOT				ENV				ENV + UV			
Crypto		**Giardia**		**Crypto**		**Giardia**		**Crypto**		**Giardia**	
log(NIP/Rot_FB)	25%	log(F)	28%	log(NIP/Rot_FB)	44%	log(NIP/Rot_FB)	45%	log(NIP/Rot_FB)	47%	log(NIP/Rot_FB)	38%
r_crypto	23%	log(S)	25%	Cfree_FB	33%	Cfree_FB_init	38%	Cfree_FB	35%	Cfree_FB_init	31%
log(F)	18%	log(Crot_FB))	24%	CFS	-8.0%	CFS	-6.8%	CFS	-8.5%	UV_reach	-9.8%
log(S)	16%	log(NIP/Rot_FB)	12%	r_crypto	5.3%	log(F)	3.9%	log(F)	4.3%	log(F)	5.2%
log(Crot_FB))	15%	V_d	6.8%	log(F)	3.6%	log(S)	3.4%	log(S)	3.7%	CFS	-5.2%
V_d	4.2%	r_crypto	3.6%	log(S)	3.4%	V_d	1.2%	log(Crot_FB))	1.6%	log(Crot_FB))	4.4%
				log(Crot_FB))	1.6%	log(Crot_FB))	0.9%	DEC	0.1%	log(S)	4.4%
				V_d	0.9%	r_crypto	0.5%	UV_reach	0.0%	V_d	1.3%
				DEC	0.1%	DEC	0.1%	r_crypto	0.0%	r_crypto	0.7%
								V_d	0.0%	DEC	0.1%

6.3.3.1 Uncertainties in modeling predation

For the environmental simulations, the most significant parameter impacting the microbial risk computed by the model is the number of IP per rotifer in the filter bed (N_{IP/Rot_FB}), followed by the concentration of (oo)cysts contaminating the filter bed at the beginning of the filtration cycle (C_{free_FB}). It is important to point out that those two variables are correlated in the model: the number of IP per rotifer (N_{IP/Rot_FB}) is computed on the basis of the (oo)cyst ingestion rate by rotifers, described by Equation (7), which directly depends on the concentration of free (oo)cysts in the filter bed. In addition, the variable *CFS*, describing the removal of (oo)cysts from raw water by physico-chemical treatment steps prior to filtration, also directly influences the concentration of (oo)cysts in the filter bed. Together, these three variables totalise a contribution of 85% to the risk of infection in environmental simulations.

We propose that the variability associated with the concentration of (oo)cysts in the filter bed originates mainly from the actual variations in (oo)cyst concentrations observed in environmental conditions rather than reflecting a lack of knowledge on the characterization of this variable, since the distribution used to describe variability of this variable (C_{RW}) is based on 350 source water parasite concentrations collected in the ICR (USEPA 2002). However, a similar statement cannot be drawn for the variable $N_{IP/Rot\_FB.}$ There is clearly some uncertainty associated to the use of Eq. (7) used to describe the ingestion and accumulation of (oo)cysts by rotifers in the filter bed: it is based on a limited set of data which were obtained in laboratory conditions with other predators than rotifers (*Paramecium*). This data comes from the only study quantifying the impact of the prey concentration on the ingestion rate of zooplankton organisms using *Cryptosporidium* oocysts (Stott et al. 2003). This approximation of the predatory behaviour of rotifers on (oo)cysts can still be thought to be realistic: *Paramecium* is known to have a similar feeding mode as some rotifers (Wetzel 2001), although it would be preferable to develop a specific relationship for rotifers feeding on (oo)cysts. In the present study, the parameter *B* in Equation (7) was derived from the data generated in a pilot-scale GAC filtration challenge test with seeded (oo)cysts of *Cryptosporidium* and *Giardia*, in order to better represent realistic ingestion conditions in granular filtration. However, high (oo)cyst concentrations were seeded in the GAC filters, which deviates from the environmental conditions where the (oo)cysts are

ingested as part of the grazing activity on the biofilm. Considering the high impact of the variable N_{IP/Rot_FB} on the final risk, it is recommended to better characterize the accumulation of IP in zooplankton organisms in granular media filters in order to sharpen the predicted risk computed by the model. We suggest that additional research under more realistic conditions would be needed to better assess the ingestion rate of predators in granular media filters.

6.3.3.2 Uncertainties in describing the transport and fate of internalized (oo)cysts

The variables *F* and *S* characterize the transport and fate of IP through granular filtration to the effluent water. Under environmental conditions, both variables are found to contribute to the output risk variability in the range of ~ 3 to 5 %. Those parameters are poorly characterized in the literature and their distribution was mostly based on the data from one pilot-scale study. Even though their impact on the risk variability was small as compared to the variable characterizing the accumulation of IP in rotifers in the filter bed, further investigation should be dedicated to improve their quantification. Since the variable *F* seems to be slightly but consistently more significant in the model output, it would be recommendable to attempt better characterizing the release of rotifers, or more generally, higher organisms in the effluent water from granular media filters, since measurements of zooplankton concentrations require less complex analytical protocols as compared to the detection of internalized pathogens. From a practical perspective, it would also be of interest to evaluate strategies which minimize the release of higher organisms. For example, filter operational conditions (e.g. velocity, or backwash frequency) or design (e.g. adding a small depth of low diameter sand media under the GAC) could represent strategies that might provide an improve control of the release of higher organisms in filtered effluents.

The variable defined with the most uncertainty though is *S*, which reflects the scarcity of the available quantitative data characterizing the occurrence of internalized pathogens in environmental conditions. Definition of this parameter could be improved by conducting further experimental work to detect internalized pathogens in water systems. In fact, quantification of *S* was solely based on the GAC pilot study measurements of internalized (oo)cysts in the filter bed and effluent water, which consists on limited data. It can be argued that the method used to

detect internalized (oo)cysts during the pilot filtration study was not 100% effective. In fact, (oo)cyst recovery rates in environmental samples are commonly low; therefore, although not characterized, the recovery of internalized (oo)cyst from granular media samples and filtered effluent water would be expected to be at best as good as the standard method using IMS and cartridge filtration. This uncertainty in analytical methods suggests a possible underestimation of the risk. Furthermore, this pilot study was preliminary with respect to the prevalence of internalized (oo)cysts. It was mostly focussed on method development and a preliminary application of these methods to find evidence for the presence of internalized (oo)cysts and their hypothesized transport to the effluent. The collected data were still limited and require further reproduction and extension.

The concentration of rotifers in the filter bed was found to have a low influence on the probability of infection by internalized (oo)cysts as computed by the model in environmental conditions (≤1.6%, except for the *Giardia* simulation including UV: 4.4%). Although concentrations of zooplankton organisms are rarely quantified in the granular media filter bed, it is not likely to vary in a very wide range. As described earlier, in this model, the distribution for this variable was defined on a range of about 2 orders of magnitude, with the highest value inspired from concentrations in the *Schmudzdecke* of slow sand filters. The case where an entire filter bed would be as densely colonized by zooplankton as the top layer of a slow sand filter is unlikely to be outranged. In this study, a focus was put on rotifers where characterizing internalization in the filter bed, since we assumed that they were the most probable vehicle for *Cryptosporidium* and *Giardia* (oo)cysts in granular media filtration. The role of zooplankton organisms such as cladocerans and nematodes in the transport or reduction of pathogens in granular filtration should be further described: some indications suggest that cladocerans such as daphnia could contribute to reducing the infectivity/viability of internalized *Cryptosporidium* and *Giardia* (oo)cysts (Connelly et al. 2007), while nematodes could transport *Cryptosporidium* oocysts in laboratory conditions and create infections in mice (Huamanchay et al. 2004), and vehicle coliform bacteria in chlorinated drinking water distribution systems following predation in granular filters (Locas et al. 2007).

It has to be mentioned that infectivity of (oo)cysts was not taken into account in this model. Firstly, about 37% of *Cryptosporidium* oocysts in the environment are estimated to be infectious (LeChevallier et al. 2003, USEPA 2005). Therefore, only a fraction of the (oo)cysts that are grazed upon by rotifers in the model should be considered in the risk assessment. This simplification leads to an overestimation of the risk by the present QMRA model, and even under those excessively severe conditions, the estimated risk is low. Secondly, the impact of grazing and internalization by rotifers on the infectivity of the (oo)cysts is unknown. In nematodes, *Cryptosporidium* oocysts have been reported to retain their infectivity and to create infection in mice (Huamanchay et al. 2004). On the other hand, *Daphnia* have been reported to significantly reduce the infectivity of *Cryptosporidium* oocysts (by ~87%) and the viability of *Giardia* cysts through the mechanisms of ingestion and excretion (Connelly et al. 2007). While Nowosad et al. (2007) detected viable *Cryptosporidium* oocysts inside rotifers sampled from natural lake waters, the infectivity of those internalized oocysts was not determined. Hence, the possible alterations to the state of IP have not been taken into consideration in the model, since no information is available on the infectivity of (oo)cysts ingested by rotifers.

6.3.3.3 Uncertainties in modeling water treatment: granular media filtration and UV disinfection efficacy

In this study, environmental simulations were performed using a 72h filtration cycle duration. Considering that accumulation of IP in rotifers was assumed to increase linearly as a function of time, it would be interesting to revisit this risk analysis for the operating conditions of slow sand filtration, which can be operated without washing/scraping of the *Schmudzdecke* for as long as a year. Also, in the case of GAC filtration, similar durations of a few hundred days can be used without backwashing when operating the filters in biological mode (Schreiber et al. 1997). It can not be stated that the modeled computation of the accumulation of IP in rotifers in the filter bed would be appropriate for such long operation times. Additionally, it can be hypothesized that a decay of the (oo)cysts through time might occur in the filter bed: Boyer et al. (2009) showed that about 20% of *Cryptosporidium* oocysts were still infectious after 12 weeks in soil columns at 10^{o}C. The possibility of such decay phenomenon was not considered in the model developed in this study.

For the environmental simulations including UV inactivation, a larger uncertainty is associated to the variable *UV_reach* than what was used in the PDF description for the simulation. The protection effect reported by Bichai et al. (2009) has not been measured directly on *Cryptosporidium* and *Giardia* (oo)cysts but on *Bacillus subtilis* spores as surrogates, and the hosts used for internalization were caused by nematodes rather than rotifers. It can be hypothesized that the percentage of the UV fluence penetrating through rotifers to reach the IP would be different than the percentage measured with nematodes. However, this could not be expressed quantitatively in the model and is subject for further study. A 2-log inactivation of nematodes has been reported to require a UV fluence of 232.5 mJ/cm^2 (Matsumoto et al. 2002). This shows a very high resistance of nematodes to UV irradiation. In fact, this can be compared to a 60 mJ/cm^2 fluence reported to achieve a 2-log inactivation of *Acanthamoeba*, which is known as a very resistant organism in its cyst form (Maya et al. 2003). Therefore, it could be hypothesized that nematodes offer a high protection to internalized pathogens against UV disinfection and could represent a worst-case in that regard. However, this remains speculation and no measurement of the resistance of rotifers against UV has been made at this time.

UV disinfection was the only additional treatment barrier simulated as it was the authors opinion that it was the only process offering a potential of lowering the microbial risk associated to internalized (oo)cysts released from granular media filters. For the common water treatment conditions prevailing in most parts of the world, chlorination would be applied after filtration to maintain a residual concentration in the distribution system. Chlorination was taken into consideration but not included in the model, since no inactivation of *Cryptosporidium* is possible and it was considered that *Giardia* cysts would be 100% protected against chlorination under typical drinking water treatment conditions. In fact, Locas et al. (2007) reported a recurrent detection of total coliform bacteria internalized in nematodes in a chlorinated distribution system (0.9-1.2 mg Cl_2/L, with > 4-hr contact time). Moreover, reports of bacteria resisting free chlorine concentrations as high as 10 mg/L for 15 minutes when internalized by nematodes (Smerda et al. 1970) provide indications that zooplankton organisms can reasonably be considered as being practically impermeable to chlorination. Similar assumptions could also be inferred for other

chemical disinfectants such as ozone, but were not taken into account in this model. According to (Ding et al. 1995), ozone was more effective than chlorine in disinfecting *E. coli* bacteria internalized by nematodes, but very little is known about the protection of internalized bacteria against ozonation. The resistance of parasites to ozone is known to be higher than that of *E. coli*, while the resistance of rotifers to ozone is unknown.

6.3.3.4 Impact of the distribution system

Finally, the risk computed in this study was based on the concentration of IP in the treated water at the exit of the treatment plant, as was done in other QMRA models taking into account the impact of water treatment processes on the infection risk (Jaidi et al. 2009). Total coliforms have been detected in nematodes in a full-scale distribution system (Locas et al. 2007). It can be discussed that the transit of internalized organisms through the distribution system may impact the risk of transmission to consumers. In fact, *Legionella*, as other bacterial pathogens, are known to develop in distribution system biofilm in association with amoebae. It is unknown whether the biofilm in drinking water distribution pipes may favour the development of human pathogens in association with other zooplankton organisms. In the case of protozoan (oo)cysts, it is unlikely that amplification will occur in the distribution network. In fact, it is anticipated that decay could lower the risk at the tap as compared to the model estimates.

6.3.3.5 Perspective on the risk estimates computed by the model

Finally, it was reassuring to calculate a mean annual risk associated to internalized (oo)cysts significantly lower than the target of 10^{-4} proposed by the USEPA. In fact, the risk associated to free (oo)cysts of *Cryptosporidium* and *Giardia* is likely to be higher than that of internalized (oo)cysts, otherwise the phenomenon of internalization by zooplankton would be raising a substantial alert related with the current water treatment practices. The risk calculation delivered by the model under environmental conditions should be viewed as a preliminary effort to better characterize the microbial risk related to internalized pathogens. Refining estimates of some key assumptions used in this model will help to adequately characterize this risk.

On-going work targeting health-related bacteria such as *E. coli* and *Campylobacter jejuni* internalized by natural zooplankton through full-scale drinking water treatment plants in Amsterdam (Netherlands) revealed no evidence of the occurrence of such internalized bacteria (Bichai, F., Barbeau, B., Rosielle, M., Baars, E., and Hijnen, W., unpublished). Results of this environmental zooplankton sampling campaign provided an indication that the detection of internalized bacteria such as *E. coli* and *C. jejuni* might be as rare as <1 in 10^5 zooplankton organisms in field conditions. Extreme concentrations of up to 71 nematodes/L were reported by (Castaldelli et al. 2005) in the effluent water of full-scale GAC filters. Even with such high zooplankton concentrations in water, the detection limit reported above leads to a calculation of <1 internalized bacteria in about 140 L or < 0.007 IP/L. Those bacteria are found in much higher concentrations in raw waters in the Netherlands as compared to *Cryptosporidium* and *Giardia* (oo)cysts, which is why they were chosen as detection targets in the environmental zooplankton sampling investigation. The probability of detecting internalized (oo)cysts in environmental water samples is likely to be even lower. It can be argued that (oo)cysts may be more resistant to digestion by some zooplankton organisms as compared to bacteria; however some pathogenic bacteria are known to be capable of infecting the gut of nematodes (Sifri et al. 2005), including pathogenic strains of *E. coli* (Caldwell et al. 2003), or to multiply inside of amoebae (Greub & Raoult 2004), including *C. jejuni* (Axelsson-Olsson et al. 2005). It has been reported that numbers between 10 – 4000 bacteria could be found as being associated to one higher organism in drinking water systems, which could include several pathogenic or opportunistic pathogens (Wolmarans et al. 2005). Clearly, the issue of predation on (oo)cysts by rotifers in granular filtration is only one aspect of the health risk that may arise from internalized organisms.

6.4 Conclusion

In this study, a conceptual QMRA model was developed in order to describe the internalization and transport of *Cryptosporidium* and *Giardia* (oo)cysts following predation by rotifers in a granular media filter. Some of the processes involved are complex and not well characterized in literature to this day. Therefore, the proposed model was developed on the basis of simplifications of those processes: (i) pathogen loading and retention in the filter bed was described as an instantaneous event; (ii) predation activity was described as an accumulation of

internalized (oo)cysts at a constant rate in predators (rotifers), which were assumed to be present in a constant concentration in the filter bed over a filter run; (iii) transport of IP to the effluent was expressed as the product of the total predator release in the effluent water and the persistence/survival of IP in rotifers. (iv) Transmission of IP through drinking water was based on the assumption of a complete resistance of IP against subsequent disinfection treatment, except for UV disinfection. Quantification of most variables was derived from laboratory and pilot-scale generated data described in literature, which was scarce in most cases.

Monte Carlo simulations were performed to compute the annual probability of infection associated to the dissemination of resistant internalized (oo)cysts through drinking water.

A sensitivity analysis revealed that the most influential variable contributing to the risk is the number of internalized pathogens per rotifer in the filter bed at the end of the filtration cycle. This aspect will require further investigation, since uncertainty is associated with zooplankton predation activities in granular media filters. The process of accumulation of IP in zooplankton organisms is not well characterized and quantified in relation with the concentration of the prey in the filter bed, and the fate of ingested (oo)cysts is still debated.

Under simulated environmental conditions, the mean annual probability of infection associated to internalized *Cryptosporidium* and *Giardia* (oo)cysts in drinking water was found to be lower than the USEPA standard of 10^{-4} infection/year, with mean values of 2.85E-06 and 2.54E-06 infections/year, respectively. The risk was significantly lowered to 2.90E-08 and 6.34E-08 infections/year for internalized *Cryptosporidium* and *Giardia* (oo)cysts, respectively, when adding a subsequent 40 mJ/cm^2 UV disinfection treatment to the filtered effluent water.

The probability of infection computed by the model should be considered as a preliminary evaluation of the risk associated with internalized (oo)cysts in drinking water, which should be refined by further investigation in environmental conditions to reduce the uncertainty associated with some of the model input variables. At this time, our previous pilot scale study stands as the only attempt to quantify internalized (oo)cysts in zooplankton organisms other than amoebae in drinking water, and more specifically in the filter bed of a granular filter. Additional quantitative

data from full-scale granular filters on the occurrence of internalized organisms and their release in the filtered water is required to optimize the proposed model.

Acknowledgements: The authors acknowledge the Industrial-NSERC Chair in Drinking Water and its industrial partners, namely the City of Montreal, John Meunier Inc., and the City of Laval for funding this research. The collaborative work with KWR Watercycle Research Institute was part of the joint research program of the Ducth Water Supply Companies. We acknowledge the support from Dr.G.M.H. Suylen (Evides) and C. Hoogendorp (Aqualab) and the assistance in laboratory work of Anke Brouwer-Hanzens (KWR) during the GAC filtration experiment referred to in this study. We acknowledge the collaboration of Yolanda Dullemont (Waternet) on the isolation and observation of zooplankton in the GAC pilot experiment. We also wish to thank Eric Baars (Waternet) and Martine Rosielle (HWL) for their important contribution to the environmental sampling work conducted in Amsterdam (the Netherlands) mentioned in the discussion.

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CHAPITRE 7 SYNTHÈSE ET DISCUSSION

Cette thèse présente un ensemble de travaux ayant permis de défricher un vaste champ de recherche, soit celui des organismes supérieurs dans l'eau potable et plus précisément, du risque microbien qu'ils peuvent représenter pour les consommateurs, un sujet jusqu'alors peu commun et peu étudié dans le domaine. Le sujet original de cette thèse a été plus spécifiquement ciblé grâce à la réalisation d'une revue de littérature critique. Certains aspects du risque microbien ont été abordés et approfondis de façon novatrice à l'aide d'études expérimentales dont l'apport scientifique comprend une portion importante de développement de méthodes en plus de la réalisation d'observations et de mesures expérimentales nouvelles. L'aspect novateur de cette thèse se trouve enfin également dans l'analyse de synthèse de sa problématique, menée dans le cadre d'un modèle de QMRA (analyse quantitative du risque microbien).

La 5^{e} publication présentée dans cette thèse constitue en quelque sorte une synthèse de l'ensemble des travaux réalisés autour de la problématique des organismes supérieurs comme facteur d'un risque sanitaire en eau potable. Le présent chapitre de discussion générale sera donc développé à la lumière de l'approche proposée dans cette analyse quantitative du risque d'infection associé aux microorganismes internalisés.

Cette analyse de risque constitue en soi une innovation dans l'étude de la problématique des organismes supérieurs en eau potable, d'abord en proposant une chaîne d'événements décrivant l'origine de l'internalisation dans la filtration granulaire, due à la prédation par le zooplancton dans ce milieu qui favorise leur prolifération. Des variables ont été définies afin de décrire le transport et la survie d'organismes internalisés par le zooplancton dans un filtre granulaire et, plus précisément, calculer la concentration d'(oo)cystes de protozoaires internalisés par des rotifères dans l'effluent des filtres granulaires. La définition de ces variables constitue un apport novateur à l'étude de la problématique de l'internalisation en proposant une approche pouvant servir de canevas pour de futures analyses de risque similaires, visant par exemple d'autres organismes internalisés (par ex. des bactéries), ou encore d'autres prédateurs que les rotifères, par exemple les nématodes.

La définition et la quantification de la plupart des variables proposées dans le modèle sont basées principalement sur l'étude de filtration à l'échelle pilote présentée au chapitre 3 de cette thèse. La protection contre la désinfection UV est basée sur l'étude expérimentale présentée au chapitre 4. Un risque très élevé de l'ordre d'une infection par 2-4 personnes annuellement a été calculé par le modèle pour les conditions de l'étude pilote, où des concentrations de l'ordre de 0.1-0.2 (oo)cystes internalisés par litre avaient été mesurées à l'effluent de filtres CAG. Dans des conditions environnementales, une probabilité d'infection annuelle de l'ordre de ~10^{-6} a été évaluée, ce qui constitue une indication préliminaire que le risque associé aux microorganismes internalisés dans l'eau potable est inférieur au risque ciblé par la réglementation de 10^{-4}. De plus, selon le modèle développé, la désinfection UV permet une réduction additionnelle d'environ 2 ordres de magnitude du risque calculé. Un raffinement du modèle et de la précision du risque calculé pourrait être gagné grâce à davantage d'investigation expérimentale sur certains aspects discutés dans les prochaines sections, soit au niveau de (i) la description de l'internalisation et du transport des microorganismes pathogènes dans la filtration granulaire, (ii) la caractérisation de l'effet protecteur associé à l'internalisation face aux procédés de désinfection et (iii) la caractérisation de l'occurrence des microorganismes pathogènes internalisés dans l'environnement.

7.1 L'internalisation des microorganismes pathogènes comme conséquence de la prédation dans la filtration granulaire des usines de production d'eau potable

7.1.1 Internalisation des microorganismes dans le lit filtrant

Dans le modèle développé dans cette thèse, il a été supposé que la filtration granulaire constituait le point de départ des événements participant au risque de transmission d'un microorganisme pathogène internalisé à un consommateur d'eau potable. Il est supposé qu'aucune source ou point de contamination ne se trouve en aval de la prise d'eau, donc aucune intrusion de microorganismes en proie à l'internalisation n'est considérée suite à la filtration. Il est également supposé que les organismes prédateurs trouvent leur origine à l'eau brute et sont sujets à une

prolifération intensifiée dans les milieux filtrants granulaires, où se trouve en plus une accumulation de la « proie ». En effet, en considérant par exemple des capacités moyennes d'enlèvement des filtres granulaires de 1.8 et 1.7 log pour *Cryptosporidium* et *Giardia* (Hijnen et Medema 2007), on peut considérer que ~98% de la concentration d'(oo)cystes à l'entrée d'un filtre granulaire est retenue dans le lit filtrant. Les probabilités d'internalisation peuvent ainsi être supposées maximales dans le lit filtrant. Il peut être discuté que l'internalisation pourrait être favorisée à nouveau ou amplifiée à d'autres étapes du traitement en aval de la filtration granulaire, par exemple dans le cas d'une chaîne de traitement comprenant plusieurs étages de filtration granulaire (par ex. filtration sur sable rapide et filtration CAG opérée en mode biologique, ou encore, comme dans le cas d'une usine d'Amsterdam, lors d'une filtration lente sur sable en toute fin de traitement). D'autres procédés dans lesquels un support matériel présente une surface favorable au développement de biofilm et d'organismes supérieurs, par exemple certains procédés d'adoucissement, pourraient éventuellement être considérés comme propices à l'internalisation de microorganismes pathogènes. Toutefois, il est raisonnable de supposer que la concentration de microorganismes « proies » serait diminuée en aval de la première filtration et donc que les probabilités d'internalisation seraient moindres aux étapes subséquentes du traitement.

La caractérisation de la concentration des (oo)cystes internalisés à l'effluent des filtres granulaires a été définie, dans l'analyse de risque présentée au chapitre 6, comme le produit de 4 variables selon l'équation suivante :

$$C_{IP\_Eff} = C_{Rot\_FB} \times N_{IP/Rot\_FB} \times S \times F$$

Une analyse de sensibilité effectuée à l'aide de Crystal Ball® a identifié la variable N_{IP/Rot_FB} comme ayant le plus grand impact sur l'évaluation du risque par le modèle développé. Cette variable représente le nombre d'(oo)cystes internalisé par rotifère dans le lit filtrant à la fin d'un cycle d'opération des filtres. La prédiction de cette variable dans des conditions de filtration granulaire dépend de la description du processus de prédation par le zooplancton. La prédation par les organismes supérieurs dans des environnements naturels aqueux a fait l'objet de plusieurs études spécialisées dans les domaines de l'écologie et de la limnologie. Les activités de prédation par les organismes supérieurs dépendent de plusieurs facteurs environnementaux dont

la température (Sander et al. 1994), la profondeur du plan d'eau (Pinel-Alloul et al. 1989), les différentes espèces composant le réseau trophique du milieu (Vaqué et al. 1994), ainsi que certaines caractéristiques des prédateurs (par ex. leur mode d'alimentation) et des proies, incluant leur taille et l'adoption de mécanismes de résistance aux stress environnementaux (dont la prédation), comme la formation de microcolonies par exemple (Matz et Kjelleberg 2005). Toutefois, pour caractériser l'ingestion de microorganismes pathogènes par le zooplancton, des études ont été réalisées pratiquement exclusivement en conditions de laboratoire. Dans de telles conditions, l'étude de Stott et al. (2003) est la seule, à notre connaissance, qui a évalué l'impact de la concentration de la proie sur le taux d'ingestion des prédateurs. Les données de cette étude ont été utilisées comme base dans le modèle pour tenir compte de la concentration des (oo)cystes dans le lit filtrant sur le taux d'internalisation des (oo)cystes par les rotifères. Les conditions « environnementales » correspondant à un lit filtrant granulaire diffèrent des conditions en eaux de surface ou de laboratoire. L'évaluation la plus représentative des conditions de prédation dans un lit filtrant se trouve probablement dans les travaux de Yolanda Dullemont (communication personnelle), où des échantillons naturels de sable ont été prélevés du *Schmudzdecke* de filtres à sable lents, puis ensemencés d'(oo)cystes de *Cryptosporidium* inactivés aux UV et marqués par fluorescence (~80 000 oocystes/ml). Des périodes de prédation entre 1.5 et 24 h à température pièce ont été allouées avant de fixer les échantillons et d'isoler les organismes du zooplancton pour observer les (oo)cystes internalisés par microscopie en épifluorescence. De tels travaux pourraient être répétés en variant la concentration de la proie afin de mieux caractériser l'impact de ce facteur sur l'internalisation en milieu granulaire. Toutefois, il est raisonnable de supposer que l'internalisation de microorganismes pathogènes en milieu naturel se produit de manière fortuite et non sélective, de sorte que la probabilité d'ingestion d'un (oo)cyste de protozoaire par un rotifère, par exemple, serait supposée équivalente à celle d'un autre microorganisme ou d'une autre particule de taille similaire à l'occasion d'une rencontre fortuite avec le prédateur. De plus, dans le contexte d'un filtre granulaire, il peut être supposé que la majorité des microorganismes pathogènes retenus dans les filtres se trouvent attachés à la surface des grains du matériau filtrant, soit par une simple adhérence ou dans un biofilm. Ainsi, il est probable que les études du comportement de prédation du zooplancton en conditions de laboratoire et en eaux naturelles ne décrivent pas de manière appropriée l'internalisation de microorganismes dans un lit filtrant, où le broutage du zooplancton a probablement lieu en surface des grains ou dans un biofilm. Il

semble que la configuration optimale pour caractériser la prédation en milieu filtrant soit dans des études pilotes permettant de reproduire des conditions réelles d'opération des filtres.

Dans l'étude pilote réalisée dans le cadre de cette thèse, des mesures d'(oo)cystes internalisés ont été effectuées à l'effluent des filtres CAG une et trois semaines après l'ensemencement des filtres. Une hausse significative des concentrations d'(oo)cystes internalisés a été observée après 3 semaines, ce qui a été attribué à une accumulation des (oo)cystes internalisés dans le lit filtrant. Toutefois, les mesures d'(oo)cystes internalisés dans le lit filtrant n'ont été effectuées qu'après 3 semaines d'opération des filtres, et donc une véritable comparaison avec les concentrations d'(oo)cystes internalisés dans le lit filtrant après une semaine n'est pas possible. Le facteur temps devrait être davantage considéré dans les études subséquentes. En effet, dans l'analyse de risque présentée au chapitre 6, l'accumulation d'(oo)cystes internalisés dans le lit filtrant a été supposée linéaire au cours d'un cycle de filtration. Il s'agit d'une hypothèse qui nécessiterait des investigations additionnelles. De plus, afin de compléter l'étude de l'internalisation dans des conditions d'opération d'usine de traitement d'eau potable, il serait pertinent d'évaluer l'impact des rétrolavages et de leur fréquence sur la concentration d'(oo)cystes internalisés dans le lit filtrant.

Enfin, il est important de rappeler que les valeurs utilisées pour quantifier l'internalisation des (oo)cystes de protozoaires dans le modèle d'analyse de risque développé ici sont basées sur une étude pionnière en filtration CAG, comportant une part importante de développement des méthodes. Cette étude rapporte d'une part un nombre limité de mesures. Ainsi, la répétition de telles mesures serait souhaitable afin d'augmenter la confiance scientifique en les valeurs de concentrations d'(oo)cystes internalisés obtenues. D'autre part, les méthodes développées dans cette étude pilote ne présentent pas de preuve visuelle directe de l'internalisation des (oo)cystes dans les rotifères, mais plutôt de fortes indications de cette internalisation. En effet, la comparaison entre les comptes d'(oo)cystes avec et sans traitement de sonication ainsi que l'optimisation du traitement de sonication permet d'attribuer l'observation d'une augmentation significative des comptes d'(oo)cystes à une internalisation très probable des (oo)cystes récupérés par sonication. L'utilisation de la sonication comme protocole permettant de récupérer des microorganismes internalisés par le zooplancton a été éprouvée de façon concluante par plusieurs

autres auteurs (King et al. 1991, Ding et al. 1995, Caldwell et al. 2003, Kenney et al. 2004, 2005, Locas et al. 2007).

L'hypothèse posée sur le rôle des rotifères dans l'internalisation et le transport des (oo)cystes de protozoaires dans l'étude pilote repose sur des observations rapportées dans la littérature ainsi que sur les travaux de Yolanda Dullemont décrits plus haut ayant, eux, permis une observation visuelle directe d'oocystes de *Cryptosporidium* internalisés par des rotifères de l'espèce *Philodina* et/ou *Rotaria*. En effet, ces espèces sont les seules à l'intérieur desquelles des oocystes fluorescents ont été observés par microscopie, excluant dans ce cas la participation d'autres organismes du zooplancton comme les cladocères, les copépodes et les nématodes dans l'ingestion et le transport des oocystes. D'ailleurs, des travaux identiques à ceux de Y. Dullemont ont été réalisés dans le cadre de cette thèse en visant spécifiquement l'identification du rôle des nématodes dans l'internalisation d'oocystes de *Cryptosporidium* dans des échantillons naturels du *Schmudzdecke* de filtres à sable lents. Dans ces travaux, une centaine de nématodes ont été prélevés un à un suite à une fixation des échantillons après 24h de prédation en présence d'une haute concentration d'oocystes (~80 000 oocystes/ml, soit la même concentration que dans les essais de Y. Dullemont). L'observation de cent nématodes au microscope en épifluorescence n'a permis de détecter aucun oocyste internalisé (résultats non publiés). Dans le cas de notre étude pilote, les (oo)cystes ensemencés dans les filtres CAG n'avaient pas été marqués par fluorescence afin d'éviter d'altérer les propriétés de surface des (oo)cystes et ainsi d'interférer avec le comportement naturel des (oo)cystes en termes d'adhérence au matériau filtrant et de transport à travers le filtre. Il serait intéressant de répéter de telles expérimentations avec un focus principal sur l'internalisation des microorganismes dans le lit filtrant en marquant les microorganismes ciblés par fluorescence. Ceci permettrait la confirmation visuelle de l'internalisation des (oo)cystes, par exemple, par les rotifères. Alternativement, la méthode FISH (hybridation fluorescente *in situ*) peut permettre d'observer des microorganismes internalisés en les marquant par fluorescence suite à leur ingestion par le zooplancton. En effet, une étape de perméabilisation dans la méthode FISH permet la pénétration de la sonde fluorescente à travers l'organisme du zooplancton pour marquer le microorganisme cible internalisé. Une telle méthode pourrait permettre d'éviter le marquage des (oo)cystes avant l'ensemencement des filtres (bien que la détection par FISH d'(oo)cystes internalisés par le zooplancton n'ait jamais été démontrée), ou encore la détection d'autres microorganismes naturellement présents à l'affluent

des filtres (non ensemencés) suite à leur internalisation, par exemple des bactéries. Toutefois, certaines limites à l'utilisation de la méthode FISH peuvent être rencontrées lors de l'analyse d'échantillons environnementaux, par exemple dans lesquels beaucoup d'autofluorescence pourrait interférer avec la détection des cibles. De plus, la méthode FISH nécessite une main d'œuvre hautement qualifiée et permet l'observation de très faibles volumes d'échantillon au microscope, ce qui nécessiterait la mise au point de méthodes de concentration efficaces des organismes du zooplancton dans lesquelles les organismes demeureraient intacts pour l'observation et l'identification au microscope.

7.1.2 Transport des microorganismes internalisés à l'effluent filtré

Deux des variables importantes caractérisant le transport et la persistance des organismes internalisés dans l'analyse de risque présentée au chapitre 6, soit les variables *F* et *S*, ont été quantifiées sur la base de l'étude pilote présentée au chapitre 3. Rappelons que la variable *F* décrit le transport des rotifères du lit filtrant à l'effluent et peut être exprimée par :

$$F = \frac{C_{Rot\_Eff}}{C_{Rot\_FB}}$$

où C_{Rot_Eff} et C_{Rot_FB} sont respectivement les concentrations de rotifères à l'effluent et dans le lit filtrant.

La variable *S* représente le taux de survie des (oo)cystes internalisés par un rotifère à travers son passage dans le lit filtrant jusqu'à sa sortie dans l'effluent filtré. Le calcul de *S* est exprimé par l'expression suivante :

$$S = \frac{N_{IP/Rot\_Eff}}{N_{IP/Rot\_FB}}$$

où N_{IP/Rot_Eff} et N_{IP/Rot_FB} sont respectivement les nombres d'(oo)cystes internalisés par rotifère à l'effluent et dans le lit filtrant.

Dans les deux cas, la quantification de ces variables basée sur les mesures de l'étude de filtration CAG à l'échelle pilote constitue une première dans la littérature. Un renforcement des indications et des mesures découlant de l'étude pilote est souhaitable pour une meilleure caractérisation du risque associé à la transmission des organismes internalisés dans l'eau potable.

Pour la quantification de la variable *F*, il serait recommandable d'établir idéalement une base de données mettant en parallèle des concentrations d'organismes supérieurs mesurées dans le lit filtrant et à l'effluent des filtres granulaires. L'énumération du zooplancton à l'effluent des filtres granulaires est relativement commune. Toutefois, très peu de mesures des concentrations d'organismes supérieurs dans le matériau granulaire des filtres sont disponibles dans la littérature. Ces mesures pourraient être effectuées de manière plus répandue selon les méthodes d'isolation du zooplancton proposées dans l'étude pilote au chapitre 3, par exemple juste avant d'effectuer le rétrolavage d'un filtre, en prélevant des échantillons de matériau granulaire à différentes profondeur du lit filtrant, ou minimalement près de la surface. Ces mesures pourraient être couplées au prélèvement en parallèle d'un échantillon à l'effluent permettant d'isoler le zooplancton, par exemple sur un filet à plancton de 30 μm, pendant (arbitrairement) les 2 dernières heures du cycle de filtration. Ces échantillons de zooplancton peuvent être fixés (par exemple à la formaldéhyde), et donc ne nécessitent pas une analyse immédiate pour le dénombrement et l'identification des organismes du zooplancton au microscope. Un tel effort d'échantillonnage du zooplancton permettrait de mieux décrire le rejet de ces organismes à l'effluent des filtres granulaires, tel qu'il a été caractérisé pour de nombreux microorganismes d'importance en eau potable.

La caractérisation de la variable *S* est plus complexe, puisqu'elle nécessite un renforcement des connaissances à plusieurs niveaux au sujet des microorganismes internalisés. En effet, une réelle compréhension de la variable *S* implique de déterminer le sort des (oo)cystes internalisés dans le lit filtrant, incluant la digestion ou l'excrétion possible d'(oo)cystes au cours du transit du rotifère jusqu'à l'effluent filtré. Ces phénomènes sont complexes et peu caractérisés dans la littérature. Leur étude nécessite un focus sur les interactions entre le prédateur et la proie à un niveau microbiologique approfondi et donc probablement des études en conditions de laboratoire permettant des observations du comportement de l'hôte et de la proie par microscopie. Sans entrer dans l'étude proprement dite de ces interactions complexes, une manière pratique de caractériser *S* a été proposée dans cette thèse, soit en comparant les concentrations d'(oo)cystes internalisés mesurées à l'effluent et dans le lit filtrant à la fin du cycle de filtration. De telles mesures pourraient être répétées grâce aux méthodes d'analyse expérimentales proposées dans notre étude pilote. Dans le modèle de QMRA développé, l'incertitude sur la variable *S* a été définie sur une échelle logarithmique en considérant une variation probable de ± 1 log autour de

la valeur de *S* mesurée dans l'étude pilote, basée sur les variations observées dans les concentrations d'(oo)cystes mesurées à l'effluent des filtres une et trois semaines après l'ensemencement. Aucune autre mesure d'(oo)cystes internalisés dans des conditions de filtration n'a été rapportée à ce jour dans la littérature. Ces mesures nécessitent effectivement la conduite d'expériences en conditions d'ensemencement des filtres, donc en conditions pilote, puisque les (oo)cystes de *Cryptosporidium* et *Giardia* sont naturellement présents en concentrations trop faibles pour effectuer de telles mesures dans des conditions d'échelle réelle.

7.1.3 Internalisation de bactéries par le zooplancton

Il semble justifié, dans l'analyse de risque présentée dans cette thèse, de supposer que les rotifères soient les principaux responsables de l'internalisation et de la transmission des (oo)cystes internalisés dans l'eau potable. Toutefois, il est raisonnable de supposer que cette hypothèse ne soit valide qu'uniquement pour les (oo)cystes de *Cryptosporidium* et *Giardia*. Il est probable que d'autres microorganismes, tels que les bactéries, soient véhiculés par d'autres organismes du zooplancton à travers la filtration granulaire et dans l'eau potable distribuée. Il ne peut toutefois pas être supposé que toutes les bactéries auraient des interactions semblables avec un hôte du zooplancton donné. Par exemple, il a été rapporté que la bactérie entéropathogène *Campylobacter jejuni* ne survit pas à son ingestion par le cladocère *Daphnia carinata* (Schallenberg 2005); toutefois, la bactérie *Vibrio cholerae* a été observée comme colonisant le tube digestif de certains copépodes et *Daphnia* sp. (Cottigham et al. 2003). Des coliformes totaux ont été détectés (par culture) à l'intérieur de nématodes dans un réseau de distribution (Locas et la. 2007). Enfin, plusieurs bactéries pathogènes humaines peuvent créer une infection et donc être amplifiées à l'intérieur du tube digestif du nématode *C. elegans*, par exemple *Salmonella typhimurium* (Aballay et al. 2000). Sifri et al. (2005) ont dressé une synthèse des nombreuses espèces de bactéries pathogènes humaines strictes ou opportunistes pouvant infecter le nématode *C. elegans* et ont défini différents groupes de bactéries selon leur comportement dans le tube digestif de *C. elegans*, incluant par exemple la création d'une infection persistante ou non dans le tube digestif, ou la libération de toxines. La littérature laisse encore à ce jour une très grande place pour l'avancée de travaux de ce genre. On ignore par exemple si ces types de comportements pathogéniques observés pour des groupes de bactéries dans *C. elegans* pourraient être étendus à d'autres espèces de nématodes incluant par exemple celles les plus communément

rencontrées dans les systèmes d'eau potable. Pour parvenir à une véritable compréhension du phénomène d'internalisation des microorganismes pathogènes dans l'eau potable, il est proposé d'envisager une tendance dans la recherche future à définir des groupes de microorganismes ayant des comportements semblables en association avec le zooplancton. Une telle approche a déjà été appliquée aux amibes dans les efforts scientifiques récents pour les décrire et les comprendre comme des hôtes et des véhicules de microorganismes pathogènes dans l'environnement. En effet, Barker et Brown (1994) ont décrit trois groupes de bactéries pathogènes humaines selon leur comportement à l'intérieur de l'amibe, soit celles qui, comme *Legionella*, se multiplient à l'intérieur de l'amibe et causent sa lyse, celles qui se multiplient sans causer la lyse de l'amibe et enfin celles qui survivent à l'intérieur de l'amibe sans toutefois s'y multiplier. Le premier de ces trois groupes est celui qui attire le plus d'attention scientifique et a été identifié comme le groupe des 'LLAP' (Legionella-*like amoebal pathogens*) parmi les 'ARB' (*amoeba-resistant bacteria*) (Greub et Raoult 2004). Il est envisageable que de telles catégories de microorganismes soient éventuellement définies par rapport à des hôtes zooplanctoniques, en commençant par les nématodes et, à titre de recommandation suite aux travaux de cette thèse, également chez les rotifères. Les nématodes sont en effet souvent rapportés comme le groupe d'organismes supérieurs dominant (en abondance) dans les systèmes d'eau potable, suivis des rotifères, ou encore en alternance avec les rotifères.

Étant donné le potentiel d'amplification de certaines bactéries pathogènes humaines rapporté chez les nématodes, des travaux futurs axés sur l'internalisation dans la filtration granulaire devraient considérer le transport de bactéries par des nématodes ou d'autres organismes supérieurs. En effet, cette possibilité d'amplification des bactéries à l'intérieur de l'hôte constitue le principal objet de l'attention scientifique consacrée au cas des amibes comme vecteurs de bactéries pathogènes. De la même manière, dans le cas des nématodes par exemple, la possibilité d'une multiplication des bactéries à l'intérieur de leur tube digestif leur confère une probabilité accrue de persistance comme organismes internalisés de manière à engendrer un plus grand risque de transmission aux consommateurs à travers l'eau potable. D'ailleurs, Wolmarans et al. (2005) ont rapporté, dans des conditions environnementales à travers une usine de traitement d'eau potable et dans le réseau de distribution, la détection de 10 à 4000 bactéries internalisées par organisme supérieur, incluant des bactéries pathogènes humaines, strictes ou opportunistes. Ainsi, l'étude de l'internalisation des bactéries, par exemple par les nématodes, pourrait permettre

la détection d'un plus grand nombre d'organismes internalisés par organisme supérieur, par rapport aux observations réalisées dans l'étude pilote portant sur l'internalisation des (oo)cystes de protozoaires, lesquels ont fort probablement un comportement inerte à l'intérieur des rotifères. Dans les conditions de cette étude pilote, simulant le pire cas de contamination d'un filtre, avec des concentrations extrêmes d'(oo)cystes ensemencés et en absence de rétrolavage pendant 3 semaines, des valeurs moyennes (± écart type) de 2.3 ± 3.2 et 4.2 ± 2.4 (oo)cystes/rotifère dans le lit filtrant pour *Cryptosporidium* et *Giardia*, respectivement, ont été calculées suite à 10 000 simulations Monte-Carlo effectuées par le modèle d'analyse de risque développé au chapitre 6. En simulant des conditions environnementales, le modèle a estimé des valeurs moyennes de $\sim 10^{-6}$ et $\sim 10^{-5}$ (oo)cystes de *Cryptosporidium* et *Giardia* par rotifère dans le lit filtrant, c'est-à-dire environ un oocyste internalisé par 1 000 000 rotifères pour *Cryptosporidium*. De telles valeurs laissent supposer que malgré un potentiel d'ingestion des (oo)cystes de protozoaires par le zooplancton, l'occurrence trop faible et possiblement les probabilités d'excrétion (ou de digestion?) trop élevées induisent un très faible risque de transmission de tels organismes internalisés dans l'eau potable. Par contre, en considérant des valeurs environnementales de l'ordre de $10^1 - 10^3$ bactéries internalisées par organisme supérieur telles que suggérées par Wolmorans et al. (2005), on pourrait supposer la transmission d'une concentration significative de bactéries internalisées dans l'eau potable. Dans le modèle de filtration développé, la variable *F* (représentant le ratio de rotifères du lit filtrant rejetés à l'effluent) a été évaluée à ~ 0.01%, ce qui réduit a priori de 4 ordres de magnitude la concentration de microorganismes internalisés à l'effluent des filtres granulaires (C_{IP_Eff}) par rapport à la concentration dans le lit filtrant selon le calcul proposé à en début de section 7.1. Toutefois, les concentrations d'organismes supérieurs à l'effluent peuvent être typiquement supposées supérieures à un organisme par litre, et souvent davantage dans le cas des nématodes. Ainsi, malgré un ratio *F* faible, si les microorganismes internalisés dans le lit filtrant persistent, voire se multiplient à l'intérieur de l'hôte dans le cas possible de bactéries pathogènes, plutôt que d'être partiellement digérées ou excrétées avant d'atteindre la sortie à l'effluent des filtres granulaires, alors leur risque de transmission pourrait être supérieur à celui calculé par le modèle d'analyse de risque développé pour les (oo)cystes de protozoaires internalisés. Il est raisonnable de supposer que c'est surtout dans le cas d'une amplification possible de l'organisme internalisé à l'intérieur de l'hôte que l'internalisation pourrait présenter un risque sanitaire potentiellement significatif à l'eau traitée.

La conduite d'une étude de filtration à l'échelle pilote telle que présentée au chapitre 3 visant les bactéries internalisées plutôt que les (oo)cystes de protozoaires présente toutefois d'autres défis méthodologiques. En effet, si les bactéries doivent être énumérées par culture, les échantillons de zooplancton isolés des filtres et de l'effluent ne peuvent être fixés. Ils doivent donc être analysés rapidement suite à leur prélèvement puisque les interactions entre les bactéries et les hôtes sont dynamiques dans les échantillons. Aussi, il existe une certaine marge de variation entre les résistances des différents organismes du zooplancton à la sonication. Dans un échantillon hétérogène, il est donc difficile de provoquer la rupture absolument simultanée de tous les organismes du zooplancton. Les bactéries étant plus sensibles que les (oo)cystes de protozoaires à la sonication, il peut être risqué d'utiliser des traitement aussi radicaux sur les échantillons dans une telle étude, puisque si certaines bactéries sont libérées de leurs hôtes avant la fin du traitement de sonication, elles pourraient être altérées par les ultrasons, ce qui pourrait interférer avec leur détection par culture. Ainsi, il pourrait être souhaitable d'envisager des méthodes plus « douces » pour l'analyse de bactéries internalisées dans des échantillons environnementaux, utilisant par exemple la microscopie, sachant toutefois que les méthodes de détection visuelles présentent aussi leurs limites, leurs interférences et leurs défis.

7.1.4 Autres perspectives de recherche sur l'internalisation dans la filtration granulaire

Suite aux travaux de cette thèse, d'éventuels travaux de recherche sont suggérés sur la filtration lente sur sable. En effet, la densité des organismes du zooplancton dans le lit filtrant, particulièrement dans le *Schmutzdecke* en surface, ainsi que l'importance de la prédation dans de tels filtres suggèrent un haut potentiel d'internalisation. De plus, les durées très longues de cycle de filtration des filtres à sable lents laissent supposer une accumulation significative d'organismes internalisés dans le lit filtrant par rapport aux cycles de filtration de 2-3 jours typiques des filtrations rapides.

Enfin, il est souhaitable que des travaux futurs dans lesquels seront réalisés des bilans de masse en filtration granulaire prennent en considération la possibilité du rôle de la prédation. En effet, bien que selon toutes probabilités la proportion de microorganismes internalisés dans un lit filtrant et à l'effluent filtré demeure faible, la prédation devrait être perçue en filtration comme un mécanisme ayant le potentiel d'influencer à la fois la performance des filtres comme barrière des

microorganismes et à la fois le transport et le rejet des microorganismes à travers le lit filtrant et dans l'effluent. Il est probable que certaines conditions favorisent un plus grand impact de la prédation dans la filtration granulaire, telles que par exemple les conditions d'études pilotes impliquant l'ensemencement de microorganismes en concentrations élevées, ou encore les études sur des longues durées de cycles de filtration.

7.2 Protection des microorganismes internalisés par le zooplancton face à la désinfection de l'eau potable

7.2.1 Désinfectants chimiques

Dans le modèle d'analyse de risque développé au chapitre 6, il a été supposé que les (oo)cystes internalisés étaient entièrement protégés contre les étapes de désinfection subséquentes, à l'exception de la désinfection UV. Étant donné la forme résistante des (oo)cystes de protozoaires et particulièrement la résistance pratiquement totale de *Cryptosporidium* à la chloration, cette hypothèse, qui est conservatrice, ne cause probablement pas une surestimation excessive du risque à l'eau traitée. Toutefois, la littérature ne contient pas à ce jour de démonstration de « l'imperméabilité » des organismes du zooplancton face à tous les désinfectants chimiques en eau potable. En effet, une protection contre la chloration a été démontrée à plusieurs reprises pour diverses bactéries internalisées à l'intérieur d'amibes ou de protozoaires ciliés (King et al. 1988, Barker et Brown 1994, Brandl et al. 2005, Snelling et al. 2005, Adékambi et al. 2006) et de nématodes (Smerda et al. 1970, Ding et al. 1995, Lupi et al. 1995, Adamo et Gealt 1996, Anderson et al. 2003, Caldwell et al. 2003). Chez l'amibe *Acanthamoeba polyphaga*, la survie de plusieurs espèces de mycobactéries internalisées a été rapportée suite à une exposition à une concentration de 15 mg/L de chlore pendant 24 heures (Adékambi et al. 2006); chez des nématodes du genre *Rhabditis*, la bactérie *E. coli* peut survivre à des *CT* pouvant atteindre plus de 2000 mg·min/L à l'intérieur du tube digestif du nématode (Adamo et Gealt 1996). Bien que la susceptibilité à la chloration de l'organisme internalisé puisse varier, des résistances à de telles doses démontrent un effet protecteur robuste des amibes et des nématodes face au chlore, ce qui peut permettre de supposer une certaine « imperméabilité » ou une efficacité comme bouclier de ces organismes supérieurs pour des microorganismes internalisés. Pour les rotifères et le zooplancton crustacéen (copépodes et cladocères), très peu d'études se sont attardées à la

protection de microorganismes internalisés face à la désinfection. Il a été rapporté que des bactéries naturellement internalisées dans le tube digestif d'organismes du zooplancton isolés de l'eau d'un lac étaient protégées face à un traitement de chloration équivalent à un *CT* de 50 mg·min/L (King et al. 1991).

En ce qui a trait aux autres désinfectants chimiques en eau potable, peu d'études ont été entreprises pour vérifier leur capacité d'atteindre les microorganismes internalisés par le zooplancton. Ding et al. (1995) ont rapporté une protection face à l'ozonation des bactéries *E. coli* internalisées par des nématodes du genre *Rhabditis*, qui sont parmi les plus communément détectés dans les réseaux de distribution d'eau potable : une telle protection a permis de récupérer 10^2-10^3 bactéries internalisées par nématode suite à une exposition à des doses de 0.45 à 1.7 mg/L d'ozone pendant 1 à 3 minutes, alors que les bactéries non internalisées étaient non détectables à de telles doses. Aucune information n'est disponible à notre connaissance sur la survie de microorganismes internalisés par des rotifères ou le zooplancton crustacéen face à l'ozonation ou le bioxyde de chlore, qui demeurent des désinfectants moins largement répandus que le chlore, dont l'utilisation est pratiquement obligatoire en Amérique du Nord pour le maintien d'un désinfectant résiduel en réseau. L'hypothèse posée sur l'inefficacité des désinfectants chimiques face aux microorganismes internalisés dans l'eau potable dans le cadre du modèle d'analyse de risque développé au chapitre 6 pourrait être à l'origine d'une surestimation du risque d'infection calculé par le modèle, dans une moindre mesure pour des microorganismes résistants tels que les (oo)cystes de protozoaires, mais dans une mesure inconnue à ce jour dans le cas de l'application du modèle à d'autres microorganismes internalisés, comme par exemple des bactéries plus sensibles à la désinfection chimique (ozone, bioxyde de chlore).

7.2.2 Désinfection UV

En ce qui a trait à la protection contre la désinfection UV des microorganismes internalisés, la seule étude disponible à ce jour est celle présentée au chapitre 4 de cette thèse. L'effet protecteur des nématodes face à la désinfection UV a été démontré dans cette étude pour deux microorganismes internalisés, soit *E. coli* et les spores de *B. subtilis*, et ce avec une reproductibilité élevée des résultats obtenus face à une fluence de 40 mJ/cm^2 typique des usines d'eau potable. L'utilisation des spores de *B. subtilis* pour évaluer la fluence effective en

désinfection UV (254 nm) selon le principe de biodosimétrie (Sommer et al. 2000), d'une part, et d'autre part, leur comportement plus stable (par rapport à *E. coli* par exemple) à l'intérieur du tube digestif des nématodes en font une cible intéressante pour l'évaluation de la capacité de pénétration du rayonnement UV à l'intérieur d'organismes supérieurs. En effet, il faut considérer que, dans le cas d'essais de désinfection UV réalisés directement avec des oocystes de *Cryptosporidium* par exemple, ces essais impliqueraient d'effectuer plusieurs étapes de manipulation avec des concentrations élevées d'oocystes infectieux et donc le besoin d'une main d'œuvre hautement qualifiée, entre autres pour les mesures d'infectivité des oocystes, et des installations conformes en termes de sécurité en laboratoire. Les spores présentent ainsi une alternative valable selon l'approche de la biodosimétrie et une simplification considérable au niveau des méthodes expérimentales. L'extrapolation de cet effet protecteur face à la désinfection UV dans le modèle d'analyse de risque développé pour des (oo)cystes internalisés par des rotifères présente toutefois une source d'incertitude. Il est en effet difficile d'évaluer si l'effet protecteur des nématodes face aux UV est équivalent à un effet protecteur associé à l'internalisation par d'autres organismes du zooplancton, tels que les rotifères. Pour une quantification plus juste de cet effet protecteur dans l'analyse de risque proposée, il est recommandable d'effectuer des essais d'inactivation UV avec des rotifères, en utilisant par exemple les spores de *B. subtilis* comme cible internalisée. Une alternative intéressante à explorer en termes de protocoles innovateurs serait l'utilisation de microsphères fluorescentes développées par Blatchley et al. (2006) au lieu de microorganismes comme cible internalisée. Ces microsphères permettent d'évaluer la dose UV reçue suite à la mesure de l'intensité de leur fluorescence. Des microsphères d'un diamètre moyen de 5.6 μm telles qu'utilisées dans la validation de systèmes de désinfection UV (Blatchley et al. 2008) sont susceptibles d'être ingérées par des rotifères, étant donnée leur taille, quoique cette hypothèse nécessite une validation expérimentale. Bien que cette idée implique nécessairement des inconnues et défis méthodologiques, le développement d'un protocole basé sur l'internalisation de ces microsphères par des organismes du zooplancton pourrait permettre d'estimer la dose UV atteignant l'intérieur de leur tube digestif et renforcer par exemple l'évaluation de l'effet protecteur mesuré sur des microorganismes internalisés tels que *E. coli* et les spores de *B. subtilis*. L'utilisation des ces microsphères fluorescentes pourraient même hypothétiquement être étendue éventuellement à l'évaluation d'autres mécanismes de protection des microorganismes face à la désinfection UV,

en reproduisant par exemple des conditions environnementales d'agrégation et d'association aux particules dans des eaux naturelles plus ou moins turbides.

Les essais de désinfection UV réalisés dans le cadre de cette thèse constituent une innovation en termes de caractérisation de l'effet protecteur des organismes supérieurs pour des microorganismes internalisés face à la désinfection UV. Le développement du protocole présenté au chapitre 4 de cette thèse est inspiré entre autre du principe d'essais similaires réalisés par Caron et al. (2007) sur l'impact de l'agrégation et l'attachement aux particules dans les eaux naturelles face à la désinfection UV. Dans les deux cas, les protocoles appliqués permettent la comparaison de l'inactivation UV pour différentes conditions (ou traitement) des microorganismes exposés à l'irradiation. Dans le cas de l'internalisation par les nématodes, l'inactivation UV est comparée pour (i) des microorganismes à l'état planctoniques (en suspension pure dans du tampon phosphate), (ii) des microorganismes en présence de débris de nématodes suite à l'extraction des microorganismes par sonication et (iii) des microorganismes contenus dans le tube digestif de nématodes. L'internalisation par des organismes supérieurs constitue un phénomène probablement beaucoup plus rare par rapport à l'agrégation et l'attachement aux particules, qui caractérisent dans une mesure plus ou moins importante la plupart des conditions environnementales des microorganismes indigènes en eaux de surface. Toutefois, l'internalisation peut être considérée comme un cas extrême d'altération à l'efficacité des procédés de désinfection due à des conditions environnementales, dont l'impact est complètement exclu lors d'essais d'inactivation sur des microorganismes issus de cultures pures en laboratoires. Une tendance dans la recherche est observée aujourd'hui vers une meilleure description des performances des procédés de désinfection en conditions réelles: en effet, le fait que la prédiction des performances des procédés de désinfection en conditions réelles est plus complexe qu'en conditions de laboratoires ne peut plus être ignoré. Ces études sur des souches pures en laboratoire, dans des conditions simplifiées, demeurent évidemment nécessaires pour connaître l'effet direct d'inactivation d'un procédé sur une cible microbienne, pour permettre de comparer l'efficacité des désinfectants entre eux, pour caractériser la susceptibilité d'un microorganisme isolé à un procédé de désinfection, pour comparer la susceptibilité à la désinfection des microorganismes entre eux de manière relativement standardisée. Toutefois, pour prédire les performances des procédés en usine de traitement d'eau potable, en conditions réelles, il est nécessaire d'étudier de plus près les comportements microbiens en conditions

naturelles, incluant les biofilms, les associations de microorganismes entre eux, aux particules et autres surfaces disponibles ainsi qu'aux organismes supérieurs.

7.2.3 Désinfection solaire

Dans le cas d'un procédé comme la désinfection solaire, la prédiction de sa performance est appuyée sur moins de littérature et suscite un peu moins de développements en recherche, lesquels sont sans doute plus disparates ou plus lents étant donné l'intérêt d'un tel procédé presque uniquement dirigé vers les communautés en développement, ce qui laisse sous-entendre peu d'intérêt de la part de l'industrie et donc moins de financement pour la recherche. En effet, en désinfection solaire, il n'est pas question de brevet ni de génération de revenus découlant de l'utilisation et de l'application du procédé. En plus, dans le cas d'une source de désinfection naturelle comme le rayonnement solaire, il faut pouvoir considérer l'impact des conditions météorologiques et climatiques dans la performance du procédé, ce qui rend son étude d'autant plus complexe et sa standardisation, plus ardue. Ainsi, encore plusieurs travaux de recherche sont en cours pour optimiser par exemple le réacteur du procédé SODIS; la caractérisation de la susceptibilité de divers microorganismes en suspensions pures face à la désinfection solaire fait également toujours l'objet de recherches. De plus, les mécanismes par lesquels les cellules des microorganismes sont endommagées par le rayonnement solaire ne sont pas encore uniformément expliqués. Peu de recherches ont été entreprises à ce jour sur l'impact des conditions naturelles environnementales dans la performance du procédé de désinfection solaire. L'impact de la turbidité, par exemple, a été le plus souvent mesuré d'une manière peu représentative des conditions environnementales, en négligeant les composantes d'agrégation et d'attachement aux particules qui interviennent dans les eaux naturelles, d'où l'intérêt et l'innovation des travaux réalisés en désinfection UVA dans le cadre de cette thèse. En plus, peu d'efforts ont été apportés en recherche jusqu'à ce jour pour tenter de tracer un parallèle entre la caractérisation de la performance des procédés de désinfection UV_{254nm} (UVC) et solaire (UVA), d'où l'aspect d'autant plus novateur des travaux présentés dans cette thèse.

Les résultats présentés au chapitre 5 comparant l'impact de mécanismes de protection face à la désinfection UVA et la désinfection UVC ont révélé des similitudes entre les deux procédés dans le cas de l'agrégation des microorganismes et de leur internalisation par les nématodes. En effet, le protocole de dispersion des agrégats a permis d'observer une augmentation du taux

d'inactivation UVC des spores de bactéries sporulantes aérobies de ~1.4 fois par rapport à leur taux d'inactivation dans une eau non traitée, selon les résultats de Caron et al. (2007), et un taux d'inactivation UVA des coliformes totaux dispersés de ~1.5 fois supérieur à celui des coliformes non dispersés. Dans le cas de l'internalisation, l'effet protecteur observé, tant en désinfection UVC que UVA, était moindre à une fluence appliquée plus faible (5 mJ/cm^2 UVC et 0.7 J/cm^2 UVA). Aux doses les plus élevées (40 mJ/cm^2 UVC et 5.6 J/cm^2 UVA), un effet protecteur comparable a été observé, avec respectivement ~16% et ~24% de la fluence appliquée atteignant les bactéries *E. coli* internalisées. Il demeure toutefois prématuré de prétendre pouvoir étendre les observations portant sur les interférences dues à de telles conditions environnementales ou mécanismes de protection des microorganismes face à la désinfection UVC à la prédiction d'une influence semblable dans la désinfection UVA. En effet, des différences ont été observées entre les deux procédés au niveau de l'impact de la présence de particules naturelles et de débris de nématodes sur l'efficacité de l'inactivation UVC et UVA. Nos travaux illustrent toutefois une variabilité considérable dans l'inactivation obtenue par l'action des radiations UVA associée à la prise en compte de conditions environnementales, suggérant un intérêt à caractériser davantage la performance de la désinfection solaire dans des eaux naturelles.

7.3 Détection de microorganismes internalisés par le zooplancton en conditions naturelles/environnementales

La caractérisation de l'occurrence de microorganismes internalisés dans la filtration granulaire a été effectuée dans des conditions d'ensemencement d'(oo)cystes de protozoaires dans des filtres à l'échelle pilote. Dans le cadre de cette thèse, des travaux additionnels ont été effectués en collaboration avec les compagnies hollandaises Waternet et KWR dans le but de détecter, en conditions complètement naturelles, des microorganismes internalisés par le zooplancton à travers des usines d'eau potable. Étant donné les trop faibles concentrations d'(oo)cystes de *Cryptosporidium* et *Giardia* dans des eaux naturelles, les bactéries *E. coli* et *Campylobacter jejuni* ont été ciblées comme microorganismes internalisés dans l'échantillonnage environnemental. Huit points d'échantillonnage ont été sélectionnés dans trois usines de traitement d'eau potable à Amsterdam (Pays-Bas), à divers stades du traitement incluant

l'effluent de filtres à sable rapides, de filtres CAG, de filtres à sable lents, de procédés d'adoucissement et d'un lac artificiel. Le zooplancton naturel a été isolé à chaque endroit à deux reprises, à deux semaines d'intervalle, au printemps, et en deux fractions basées sur la taille des organismes, en utilisant des filets à plancton de 10 μm et 30 μm, le filet le plus fin visant la rétention des amibes. Un protocole analytique basé sur la chloration pour l'élimination des bactéries non internalisées et un traitement de sonication optimisé pour la rupture des organismes du zooplancton a été utilisé pour détecter les bactéries internalisées dans les échantillons environnementaux. En considérant tous les échantillons analysés au cours de cette campagne d'échantillonnage, un total d'environ 100 000 organismes du zooplancton ont été isolés pour la détection de bactéries internalisées, incluant ~31 000 amibes, 38 000 rotifères et 23 000 nématodes en tant que groupes dominants, avec des quantités moindres de copépodes et de protozoaires ciliés. Aucune bactérie internalisée n'a été détectée dans l'ensemble des échantillons analysés, ce qui suggère que l'occurrence de bactéries *E. coli* et *C. jejuni* internalisées peut être un événement aussi rare que <1 dans 10^5 organismes du zooplancton dans des conditions réelles. Il est toutefois aussi possible que la non détection de bactéries internalisées au cours de l'échantillonnage environnemental soit associée à une faille dans le protocole analytique, comme il est possible que les conditions saisonnières au moment de l'échantillonnage soient moins propices à la détection de bactéries internalisées par rapport à d'autres époques de l'année où la prolifération de zooplancton est plus abondante (en eau chaude par exemple) ou lorsque des événements naturels engendrent de plus hautes concentrations de bactéries aux points d'échantillonnages choisis. Ainsi, ces résultats préliminaires peuvent être interprétés ou bien comme l'indication d'une occurrence très faible de microorganismes pathogènes internalisés dans les systèmes d'eau potable, ou encore d'une difficulté élevée à les détecter, ce qui semble très probable également. En effet, des conditions sévères de chloration et de sonication telles qu'appliquées dans un tel protocole analytique pourraient par exemple nuire à la détection des bactéries internalisées, surtout si elles sont présentes à de faibles niveaux dans les échantillons naturels. Aussi, en ciblant un groupe beaucoup plus large de bactéries, Wolmarans et al. (2005) ont pu détecter des hautes concentrations de bactéries internalisées par le zooplancton dans des conditions d'usine d'eau potable à échelle réelles.

Ainsi, une première recommandation à ce niveau serait de vérifier l'adéquation des méthodes analytiques comportant des étapes de traitement pouvant endommager les bactéries présentes

dans les échantillons, en comparaison par exemple avec des étapes de rinçage des échantillons à l'eau stérile (en substitution à la chloration pour éliminer les bactéries non internalisées) ou des méthodes de détection visuelle des microorganismes internalisés. Ensuite, il pourrait être utile de réaliser un tel échantillonnage dans des conditions où les probabilités d'internalisation sont maximisées, soit par exemple dans des eaux usées. En supposant qu'un risque sanitaire dû à l'internalisation de microorganismes pathogènes dans l'eau potable soit probable surtout dans le cas d'une amplification possible de ceux-ci à l'intérieur d'organismes supérieurs, comme dans le cas des amibes, l'analyse d'échantillons d'eaux usées prélevés à des endroits stratégiques pourrait proposer une réponse au moins préliminaire à la question suivante : « Quelle est la probabilité qu'une amplification des bactéries internalisées dans le zooplancton (par exemple des nématodes) se produise dans des conditions naturelles? ». Si un tel phénomène n'est pas détectable dans des eaux hautement contaminées, il est peu probable qu'il se produise à un niveau détectable en eau potable, et conséquemment, il est peu probable qu'il soit associé à un risque sanitaire significatif pour les consommateurs.

Il demeure que certains endroits peuvent être critiques au niveau de l'internalisation de microorganismes pathogènes en eau potable. Dans cette thèse, la filtration granulaire a été identifiée et analysée comme l'endroit le plus susceptible de produire une concentration significative de microorganismes pathogènes internalisés dans l'eau potable. Toutefois, des coliformes totaux internalisés par des nématodes ont pu être détectés en conditions naturelles dans un réseau de distribution (Locas et al. 2007). Les réseaux de distribution sont souvent ciblés comme étant associés à la présence d'organismes du zooplancton (Chang et al. 1960*b*, Levy et al. 1986, van Lieverloo et al. 1998). Il serait également pertinent d'étudier l'impact du réseau de distribution, considérant la présence de biofilm, sur une prolifération possible de bactéries internalisées. Enfin, un échantillonnage environnemental pour détecter des microorganismes internalisés dans les systèmes d'eau potable pourrait être répété avec des méthodes de détection optimisées et adaptées aux conditions environnementales d'eaux plus contaminées et en ciblant davantage les endroits où une potentielle intensification de l'internalisation est soupçonnée, incluant par exemple des procédés dans lesquels le développement de biofilm est observé.

Enfin, le premier indice du rôle potentiel des organismes supérieurs dans la qualité microbiologique de l'eau potable demeure sans doute à ce jour les observations rapportées dans la littérature (ou souvent vraisemblablement non rapportées) d'une détection ou d'une persistance

inattendue et inexpliquée d'un microorganisme pathogène ou indicateur dans un système d'eau potable, comme dans le cas étudié par Locas et al. (2007), ou encore par exemple dans le cas d'une sous-performance d'un procédé de désinfection en usine. La principale retombée espérée des travaux de cette thèse serait d'amener le rôle des organismes supérieurs à être examiné comme une possible explication de tels scénarios pouvant se présenter de manière accidentelle mais probablement plus fréquente qu'on ne le croit. Ces phénomènes constituent probablement une clé importante de l'investigation sur l'occurrence et l'importance sanitaire potentielle des microorganismes internalisés dans l'eau potable.

CONCLUSION ET RECOMMANDATIONS

Les travaux réalisés dans le cadre de cette thèse ont permis d'apporter les conclusions suivantes :

(1) Dans des conditions d'ensemencement d'(oo)cystes de protozoaires *Cryptosporidium* et *Giardia* dans un filtre au charbon actif granulaire (CAG), la prédation par le zooplancton colonisant naturellement le lit filtrant peut résulter en l'internalisation d'une fraction limitée des (oo)cystes dans le lit filtrant, suivie du transport d'une portion des (oo)cystes internalisés à l'effluent. Une hausse de la concentration d'(oo)cystes internalisés à l'effluent filtré a été observée 3 semaines après l'ensemencement des filtres en l'absence de rétrolavage, suggérant une accumulation des (oo)cystes internalisés par prédation dans le lit filtrant au cours du cycle de filtration. Les rotifères sont suggérés comme principaux responsables du transport des (oo)cystes internalisés à l'effluent.

(2) Les microorganismes internalisés par des organismes supérieurs peuvent être partiellement protégés contre la désinfection UV (254 nm). Un protocole de sonication optimisé pour la destruction des nématodes et l'extraction des bactéries internalisées a permis de démontrer qu'environ 15-16% de la fluence de 40 mJ/cm^2, typiquement appliquée dans les usines de traitement d'eau potable, atteint les bactéries *E. coli* et les spores de *B. subtilis* exposés à la désinfection UV lorsqu'internalisés par des nématodes *C. elegans*. Cet effet protecteur est moins accentué face à une fluence plus faible appliquée. Une inactivation de 2.7 log de *E. coli* et 0.7 log des spores de *B. subtilis* internalisés a été atteinte lors de l'application d'une fluence de 40 mJ/cm^2. Ainsi, la désinfection UV présente un potentiel d'inactivation des microorganismes internalisés par des organismes supérieurs.

(3) Un effet protecteur comparable dû à l'internalisation par les nématodes face à la désinfection solaire (UVA) a été observé en appliquant un protocole identique à celui développé pour les essais sur l'impact de l'internalisation face à la désinfection UV. En effet, environ 24% d'une fluence UVA de 5.60 J/cm^2 a été estimée comme effective pour inactiver les bactéries *E. coli* localisées à l'intérieur de nématodes *C. elegans*. À cette fluence, une inactivation de près de 4 log a été mesurée pour des bactéries *E. coli*

planctoniques (libres), alors que moins d'un log a été mesuré pour les bactéries internalisées. Comme en désinfection UV (254 nm), une protection moins prononcée est observée à une fluence UVA plus faible. Parallèlement, une réduction du taux d'inactivation des coliformes totaux par les UVA due à l'agrégation des microorganismes entre eux a été démontrée, trouvant un effet protecteur semblable en désinfection UV et solaire dû à l'agrégation naturelle des microorganismes dans les eaux de surface. Toutefois, l'enlèvement des particules par une filtration membranaire de 8 μm n'a démontré aucun impact sur l'efficacité de l'inactivation par les UVA, contrairement à la hausse du taux d'inactivation démontrée par un protocole identique face à l'inactivation UV à 254 nm.

(4) Une analyse quantitative du risque microbien (QMRA) a permis d'estimer que le risque annuel d'infection chez les consommateurs d'eau potable dû à l'ingestion d'(oo)cystes de protozoaires internalisés par des organismes supérieurs est inférieur au risque permis d'une infection par 10 000 personnes annuellement. L'application d'un procédé de désinfection UV en aval de la filtration granulaire dans les usines de traitement d'eau potable a été évaluée comme pouvant réduire d'environ 2 ordres de magnitude la probabilité d'infection associée aux (oo)cystes internalisés dans l'eau potable.

La recherche présentée dans cette thèse est constituée d'études pionnières à plusieurs niveaux pour le domaine de l'eau potable. D'une revue de littérature exhaustive à la construction d'un modèle d'analyse de risque, en passant par le développement de méthodes en laboratoire et des mesures expérimentales contribuant à décrire le phénomène d'internalisation, ces travaux ont permis de mettre en lumière plusieurs pistes de recherche gagnant à être approfondies afin de renforcer la réponse proposée à la question « Quel est le risque sanitaire associé aux microorganismes pathogènes internalisés par des organismes supérieurs dans l'eau potable ? ».

Bien que, suite aux travaux de cette thèse, le risque sanitaire associé aux microorganismes internalisés dans l'eau potable soit supposé faible, plusieurs aspects de la question gagneraient encore à être davantage élucidés. Tout d'abord, rappelons que les mesures d'organismes internalisés présentées dans l'étude de filtration à échelle pilote fournissent des indications et des données quantitatives nouvelles, mais qui demandent à être renforcées par des mesures

expérimentales similaires. Il est suggéré d'envisager de telles études au niveau de la filtration lente, qui présente un haut potentiel d'internalisation dû à la densité du zooplancton dans la partie supérieure du lit filtrant et l'importance de la prédation dans ce type de filtration granulaire. De plus, l'identification visuelle des organismes du zooplancton contenant des microorganismes internalisés est souhaitable pour confirmer, par exemple, le rôle des rotifères dans le transport des (oo)cystes de protozoaires dans la filtration granulaire. Enfin, les travaux réalisés à l'échelle pilote suggèrent de prendre en considération la possibilité du rôle de la prédation dans la réalisation de bilans de masse dans de futures études en filtration granulaire.

Au niveau des interactions entre les organismes hôtes et les microorganismes pathogènes internalisés, il est suggéré de tenter une catégorisation des microorganismes ayant des comportements semblables en association avec le zooplancton, d'une manière similaire aux groupes qui ont été définis par rapport à leur comportement à l'intérieur d'amibes. Ainsi, tel que discuté au chapitre précédent, certaines bactéries présentent la capacité de créer une infection dans le tube digestif d'un hôte alors que d'autres bactéries seront probablement digérées ou seulement transportées à l'intérieur de l'hôte sans s'y multiplier. Dans le cas d'(oo)cystes de protozoaires, la capacité d'organismes du zooplancton à les digérer n'a jamais été démontrée; il est plus probable que les (oo)cystes se comportent comme des particules inertes à l'intérieur de l'hôte qui seront excrétées, ou encore qui peuvent s'accumuler à l'intérieur du tube digestif.

Des travaux futurs devraient également considérer une étude plus approfondie du risque associé au transport de bactéries par des nématodes ou d'autres organismes supérieurs dans des conditions environnementales, avec un focus sur les systèmes biologiques hôte-pathogène présentant un potentiel d'amplification des bactéries dans le tube digestif de l'hôte. En effet, une telle capacité d'amplification des microorganismes internalisés suggère un risque sanitaire potentiellement plus élevé que celui évalué dans cette thèse pour des (oo)cystes de protozoaires internalisés. La conduite d'échantillonnages environnementaux visant la détection de microorganismes naturellement internalisés par le zooplancton est souhaitable, avec des protocoles optimisés, afin d'avoir un aperçu réaliste de l'occurrence de ce phénomène dans les systèmes d'eau potable et renforcer les estimations du risque fournies par le modèle développé dans cette thèse.

Enfin, au niveau de l’industrie de l’eau potable, il est suggéré, suite aux travaux de cette thèse, de considérer le rôle potentiel des organismes supérieurs dans le transport et la protection de microorganismes internalisés comme pouvant être à l’origine d’une détection occasionnelle ou d’une persistance inattendue d’un microorganisme pathogène ou indicateur à certaines étapes du traitement en usine ou encore en réseau de distribution.

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